Association of herpes simplex virus type 2 with cervical carcinoma.

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Association Of

SIMPLEX VIRUS

Herpes Type 2 With Cervical Carcinoma

A thesis submitted for the degree of Ph.D. in the University of London.

by

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"BE CAREFUL WHEN YOU ARE LOOKING FOR A THING,
OR YOU WILL BE SURE TO FIND IT."

LOUIS PASTEUR

To

Lalith, Shashikala and Rasika.

ABSTRACT

ASSOCIATION OF HERPES SIMPLEX TYPE 2 WITH CERVICAL CARCINOMA

Lalitha N. Mendis

Sera obtained from patients with invasive cervical carcinoma (Britain, Malawi, Sri Lanka and Sudan), with dysplasia and carcinoma *in situ* (Britain), from matched controls, from patients with a variety of other malignancies and from patients with herpetic infection were studied.

Sera were tested by indirect immunofluorescence for IgG and IgA antibodies to membrane antigens (MA), and IgA antibodies to virus capsid antigen (VCA) of HSV-2 infected cells. Complement fixation (CF) and enzyme linked immunoabsorbent assay (ELISA) for HSV-1 and HSV-2 specific antibody were also carried out. Levels of total IgG and IgA and carcinoembryonic antigen (CEA) were also determined.

Compared with controls and patients with non-squamous malignancies, IgA anti-MA was detected in higher titres in a significantly higher proportion of patients with cervical anaplasia (dysplasia, carcinoma *in situ*, and invasive carcinoma), patients with non-genital squamous carcinoma and patients with genital herpes. Neither the prevalence nor titres of IgA anti-VCA differentiated patients with cervical anaplasia from controls.

Evidence is presented which suggests that the high prevalence of IgA anti-MA in patients with non-genital squamous carcinoma may be due to the presence of a cross reacting antigen between HSV-2 infected cells and squamous carcinoma cells.

IgA anti-MA persisted in patients with invasive carcinoma long after treatment as well as in patients with non-recurrent genital herpes long after the initial attack. In contrast, in patients with genital herpes IgA anti-VCA was transient and detectable only for a short period following clinical infection.

Although the prevalence of HSV-2 specific antibody in patients with cervical anaplasia and controls showed the virus was associated with cervical carcinoma in Britain, Malawi and Sri Lanka, evidence to show that cervical carcinoma may be of multiple aetiology is discussed.

Following treatment for cervical carcinoma, a rise in total IgG and IgG anti-MA was associated with tumor recurrences. Levels of CEA were not found to be of diagnostic or prognostic value.

ELISA was more sensitive than CF in detecting HSV-1 and HSV-2 specific antibody. The IgA anti-MA response was more type specific than the IgG anti-MA response.

Findings relevant to the cervical screening programme in the U.K. are discussed.

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Chapter 1

HISTORICAL SURVEY

CHAPTER 1

HISTORICAL SURVEY

Three main streams of observations and techniques contributed to the linking of HSV-2 with cervical carcinoma.

A. Clinical and virological;

B. Epidemiological;

C. Cytological.

A. CLINICAL AND VIROLOGICAL OBSERVATIONS THAT LED TO THE LINKING OF HSV-2 TO CERVICAL CARCINOMA

According to Beswick (1962), the word Herpes has been used over a period of 25 Centuries as a medical term to describe a variety of lesions. In the Prorrhetics and coan Prognostications which date to pre-Hippocratic (430 BC) times, it was used respectively to describe serpiginous ulcers and lesions which could have been caused by herpes zoster. The physician Herrodotus (AD 100) gave a very accurate clinical description of herpes labialis (febrilis) but did not use the term herpes. Galen (AD 131-201) applied the term to almost any ulcerative condition of the skin and Actuarius (12th or 13th Century) to cutaneous ring-worm. Interestingly the description of a lesion resembling a herpetic infection, including its site of occurence came from Shakespeare, in the lines by Mercutio in Romeo and Juliet (Act I. Scene IV).

> "O'er ladies lips who straight on kisses dream
> which oft the angry Mab with blisters plagues
> Because their breaths with sweetmeats tainted are."

However it is Mab, the fairies' angry mid-wife, who has been credited with the ability to cause these lesions, and for no greater crime than that "their breaths with sweet-meats tainter were."!

Richard Morton (17th Century) described fevers accompanied by herpes but did not specify the site of eruption. Later, Daniel Turner (18th Century) also stressed the predilection of herpes for the face, and recognised both zoster and ring-worm as different forms of herpes. The classification of ring-worm as a herpetic lesion prevailed up to the mid-19th Century. Robert Williams (early 19th Century) described five species of the genus herpes of which H. zoster, H. labialis and H. praeputialis are the same as the conditions these terms describe today. Finney and Pringle (late 19th Century) recognised two species of herpes, herpes catarrhalis and herpes zoster, the former being subdivided into herpes facialis and herpes progenitalis. Herpes gestationis, i.e. herpes associated with pregnancy, was described by Gibert in 1840.

The first classical decription of genital herpes was recorded in 1736 by the French physician Jean Astruc (cited in Hutfield, 1966). He described the lesions as being "several hydatids, or watery and crystalline bladders which are filled with lymph that is thin, or thick, opaque or diaphanous, alone or mixed with air" and seen in both men and women - "These disorders are proper to men alone but (mutatis mutandis) are common to women from the same cause." The sites at which he observed lesions in women were

"the labia pudendi, nymphae, clitoris, and its prepuce, as also the caranculae myrtiformes at the orifice of the vagina" and "focal ulcers on the anus by the unnatural use of venery." The histology of the herpes genitalis vescicle was first described by Unna in 1883 (cited in Hutfield, 1966). In addition, having studied 200 cases of herpes progenitalis over a four-year period, he observed that it "was a very frequent malady" in women of a "particular class" and was a "vocational disease" of women rather than men although both sexes were susceptible. Herpetic ulceration of the cervix was also noticed in the latter part of the 19th Century and Rollet (1869) first implicated it as a cause of vaginal discharge. The virus was first isolated from lesions in the eye onto rabbit corneal tissue by Gruter (1924), and for the first time from three cases of vulvo vaginitis by Slavin and Gavett in 1946. This isolation too was made on rabbit cornea. By the mid-1960s the clinical condition and its aetiological agent, the herpes simplex virus type 2, were well documented. The findings of Slavin and Gavett were confirmed in three more cases of vulvo vaginitis (Lazar, 1955) and involvement of the cervix coincident with vulvo vaginitis (Dooley et al, 1957; Yen et al, 1965) and as an isolated cervicitis (Nigogosyan and Mills, 1965) were described. Evidence that the cervix is a major site of genital infection in the female was shown by Josey et al in 1966. From the descriptions of genital herpes by Jean Astruc and Unna, it is clear that they classified it as a venereal disease. Direct evidence of venereal transmission of the virus was shown by clinical diagnosis and virological isolation from sexual contacts of infected consorts

(Nahmias et al, 1969; Rawls et al, 1971). As Kessler (1976) has pointed out, it was not possible to distinguish without a doubt at the time whether these post coital attacks of HSV-2 infection were primary attacks (brought on by venereal transmission) or recurrent attacks occurring in people with endogenous latent herpes virus. These questions can now be answered with the development of restriction enzyme technology as demonstrated by the work of Buchman et al (1978) and Lonsdale et al (1978). By this technique different variants of the virus within the two main serotypes are recognised by the cleavage patterns produced in their nucleic acids by various bacterial endonucleases. These patterns persist after the virus is propagated in the laboratory. As an epidemiologic tool this technique is analogous to phage typing in bacteriology.

B. EPIDEMIOLOGICAL OBSERVATIONS THAT LED TO THE LINKING OF HSV-2 TO CERVICAL CARCINOMA

(a) The role of coitus

The most important and fundamental epidemiological clue in cervical carcinoma is the freedom of the virgin woman from squamous carcinoma. Domonico Rigoni Sterne, the chief physician of the Verona Hospital (Rigoni Sterne, 1842), concluded a study on cloistered nuns and married women, from which he concluded that the susceptibility of the uterus to cervical cancer in the two groups varied according to "the natural exercise of its function." This was confirmed by several subsequent studies (Table 1.1). Precise information on the histology of the rare cases of cervical

Table 1.1 Studies which examined the incidence of cervical carcinoma among celibate women.

REFERENCE	STUDY GROUP	OBSERVATIONS
Gagnon (1950) in Quebec	a) Medical records over a 20 year period of approx 13,000 nuns representing in civilian life a population of 65,000 people.	Expected number of cases 72. Observed number - none.
	b) Approx. 3,230 nuns over a 20 year period.	No cases of cervical carcinoma.
Towne (1955) Chicago	a) 574 women with carcinoma of the cervix.	37 (6.4%) were unmarried 3 were nuns (0.52%).
	b) 20 year survey of convents with a total annual average of 10,000 women.	3 cases of carcinoma of the cervix detected.
Taylor (1959)	The medical records of 2,700 Catholic nuns in the USA belonging to 3 orders.	3 cases of carcinoma of the cervix. The total rate of cancer of the genital organs in nuns calculated to be 22% lower than that for all white women.
Rotkin (1967b)	416 patients with carcinoma of the cervix.	All had experienced heterosexual contact.

carcinoma occurring in nuns (Taylor et al, 1955; Towne, 1955 in Table 1.1), i.e. whether they were squamous carcinomas or adenocarcinomas, is not available. This information is important as the epidemiology and aetiology of adenocarcinomas of the cervix with respect to coital factors is different to squamous carcinomas (Martin, 1967).

(b) Age at first coitus

Early age of onset of sexual activity stands out as the single most important coital factor associated with the development of carcinoma of the cervix (Wynder et al, 1954; Rotkin, 1967a and 1967b; Stewart et al, 1966; Boyd and Doll, 1964; Christopherson and Parker, 1964).

(c) Number of coital partners and coital frequency

Evidence is suggestive that multiple coital partners is also an important risk factor (Rotkin, 1973). Studies which have examined the influence of coital frequency are contradictory, some showing a positive relationship (Boyd and Doll, 1964), others not (Rotkin, 1967; Stewart et al, 1966; Jones et al, 1958).

(d) Prostitution and venereal diseases

Prostitutes have been found to carry a four to six times greater risk of developing cervical carcinoma than women of comparable socioeconomic groups (Rojel, 1953; Pereyra, 1961). A high frequency of previous or concurrent venereal disease has been found in women with cervical carcinoma (Levin et al, 1942; Jones et al, 1958; Segi et al, 1957; Moghissi et al, 1968).

(e) Socioeconomic factors

Studies in a number of countries have shown cervical carcinoma to be more common in women belonging to low income groups (England and Wales - Kennaway, 1948; United States - Dorn and Cutler, 1959; China - Li, 1959; Denmark - Clemmeson, 1951; Jamaica - Cummins, 1960). Suggestions offered to explain this increased incidence include (i) nutritional deficiency (Wynder, 1968 - subclinical vitamin A deficiency), (ii) rapid sexual maturity, i.e. early initiation of coitus/marriage (Christopherson, 1965), (iii) poor obstetric care leading to higher incidence of lacerated and ulcerated cervices (Lombard and Potter, 1950; Smith, 1941), and (iv) a higher protamine to histone ratio in sperm head proteins (Reid et al, 1978). The freqency of genital herpes has been shown to be higher among lower social classes than upper (Ng et al, 1970) and highest among patients seen in venereal disease clinics (Skinner, 1976).

C. CYTOLOGICAL OBSERVATIONS AND TECHNIQUES WHICH CONTRIBUTED TO THE LINKING OF HSV-2 TO CERVICAL CARCINOMA

These cytological observations fall into two broad categories:

(a) Studies which described the cellular manifestations of viral infection, and

(b) those correlating the changes in exfoliated cervical cells with the histological diagnosis of cancer.

(a) Cytological manifestations of viral infection

The intranuclear inclusions of herpes were first

described by Lipschutz in 1921. In 1949 Blank demonstrated how Giemsa stained smears of fluid from skin vesicles could differentiate herpes simplex, zoster and varicella from other bullous lesions. Varga and Browell (1960) reported viral inclusion bodies in the vaginal smears taken from 11 women with vaginal erosion. The agent from such vaginal lesions was identified as being HSV-2 by Sterne and Longo in 1963 following the detection of multi-nucleate cells and type A inclusions in the vaginal smears. The specificity of these cytological changes for the HSV-2 virus was demonstrated later by human studies as well as studies on mice genitally inoculated with HSV-2 (Naib et al, 1973). In these studies the sensitivity of the cytological techniques for identifying HSV-2 infection was compared with virological isolation and found to be of the order of 65-75%.

(b) Exfoliated cervical cell cytology

In 1943 Papanicolaou and Traut described a method for diagnosing uterine cancer by the study of exfoliated cells seen in a vaginal aspirate from the posterior vaginal fornix. Ayre (1947) suggested the use of a spatula to scrape the cervix in the region of the squamocolumnar junction and it was found that dysplasia and carcinoma in situ produced cells that could be recognised as abnormal and that sub-clinical invasive carcinoma could be discovered in an apparently healthy cervix by a cytological smear. This method furnished a useful means of studying the pathogenesis, epidemiology and aetiology of cervical carcinoma more

Table 1.2 Studies reviewed in Stanley (1958) which proved the viral aetiology of some malignancies seen in animals.

REFERENCE	HOST	TUMOR
Ellerman & Bang (1908)	Chicken	Leukaemia
Rous (1911)	Chicken	Sarcoma
Shope (1932)	Rabbits	Fibroma
Shope (1933)	Rabbits	Papilloma
Lucké (1934)	Frogs (Rana pipiens)	Adenocarcinoma
Bittner (1936)	Mice	Breast carcinoma
Gross (1951) Friend (1957)	Mice	Leukaemia

extensively. Furthermore, by the use of this technique, cervical carcinoma became the first cancer to be diagnosed before the appearance of clinical signs and symptoms.

Evidence for the viral aetiology of some animal malignancies had accumulated from the turn of the century (Table 1.2). Against this background it did not seem impossible that two diseases, i.e. genital herpes of proven viral aetiology and cervical carcinoma, both with similar epidemiology and involving the same target organ should be linked. Thus the three streams of independent observations (page 1) converged in and contributed to the first study linking HSV-2 to cervical carcinoma which was conducted by Naib, Nahmias and Josey in 1966 at the Emory University School of Medicine in Atlanta, Georgia, USA. In this study, 62 of approximately 40,000 patients screened for malignancy by vaginal smears showed cellular changes compatible with herpes simplex infection. On histologic examination of cervical biopsy material from 32 of these 62 patients, four patients were found to have carcinoma <u>in situ</u>, six patients dysplasia and 11 had acute cervicitis - i.e. 10/62 (16%) of patients developed cervical anaplasia (used to denote dysplasia, carcinoma <u>in situ</u> and invasive carcinoma). Similar studies carried out later demonstrated subsequent development of cervical anaplasia in a higher proportion of women with genital herpes (studies 1 and 2 in Table 1.3), whereas in others the proportion was much lower (studies 3-6, Table 1.3). The incidence of cervical anaplasia in a control population was determined only in study 1 and was 1.6% (4/245). The number that regressed spontaneously to normal cervical

PROSPECTIVE STUDIES

Table 1.3 Studies showing the association of cytologically detected genital herpes and cervical anaplasia.

REFERENCE	NO. OF PATIENTS WITH GENITAL HERPES	NO. OF CONTROLS WITH NO EVIDENCE OF GENITAL HERPES	NO. WHO DEVELOPED ANAPLASIA (%)	ANAPLASIA DETECTED BEFORE EVIDENCE OF GEN. HERPES WAS NOTICED	ANAPLASIA DETECTED AT TIME GEN. HERPES WAS NOTICED	ANAPLASIA DETECTED AFTER GEN. HERPES WAS NOTICED
1. Naib et al (1969)	245	245	58 (23.7%) 4 (1.6%)	10 (1 month-3 prior)	22	26 (1 month-49 after)*
2. Naib et al (1973)	673	No controls	105 (15.6%)	23 <1 yr. prior		32
3. Ng et al (1970)	256	No controls	24 (9%)	18 (14 regressed spontaneously)		6 (2-3 yrs. after)
4. Jordan et al (1972)	43	No controls	4 (10%)			4
5. Wolinska et al (1970)	37	No controls	2 (5%)	2		

Table 1.3 (cont'd)

REFERENCE	NO. OF PATIENTS WITH GENITAL HERPES	NO. OF CONTROLS WITH NO EVIDENCE OF GENITAL HERPES	NO. WHO DEVELOPED ANAPLASIA (%)	ANAPLASIA DETECTED BEFORE EVIDENCE OF GEN. HERPES WAS NOTICED	ANAPLASIA DETECTED AT TIME GEN. HERPES WAS NOTICED	ANAPLASIA DETECTED AFTER GEN. HERPES WAS NOTICED
6. An (1969)	18	No controls	2 (11%)			

* included 3 cases of carcinoma in situ whose biopsies were taken 4 months, 14 months and 31 months after detection of viral infection, and 5 cases of dysplasia which developed 3 years following cytological diagnosis of genital herpes.

cytology was mentioned only byNg et al (1970) (study 3). Thus, although these studies were suggestive of a positive correlation between genital herpes and cervical anaplasia, they lacked proper controls and sufficient follow-up cytology for firm conclusions to be drawn. As far as is known, no further prospective studies of patients with virologically- or cytologically-confirmed genital herpes has been reported since. Thereafter, the emphasis of research shifted to retrospective seroepidemiologic studies in a number of countries (Table 1.4). Although they yielded quicker results they failed to yield precise information for reasons discussed below.

The studies listed in Table 1.4 varied in the herpes virus strain used and the serologic technique employed. Of the nine assays listed in the table, the plaque reduction test, the microneutralization test and the kinetic neutralization test, were compared by Rawls et al (1970a) and found to agree in 83% to 89% of the determinations. When compared with controls, a higher proportion of women with cervical anaplasia had antibody patterns which were thought to represent previous infection with HSV-2, with the exception of patients with cervical anaplasia in study no. 28 (Israel), no. 18 (Formosa), no. 26 (Colombia) and no. 20 (Japan). The sera of patients in studies 28, 18 and 26 and in no. 30 (New Zealand) were tested by the same technique and laboratory in Houston, USA as sera in study no. 24 (Uganda), in studies no. 5 and 6 (Negro and Caucasian patients in Houston, USA) and no. 7 (Caucasian patients) in Virginia, USA. Since in studies 5, 6 and 7 a significantly

higher proportion of patients with cervical carcinoma were shown to possess HSV-2 antibodies, the lack of similar results among patients from Israel, Taiwan and Colombia cannot be attributed to difference in laboratory or technique. The results on sera from New Zealand (study 30) were in agreement with results observed in New Zealand sera in a previous study (no. 31) - i.e. the proportion of invasive carcinoma patients with HSV-2 antibodies was low and not much higher than the proportion of controls having HSV-2 antibodies. Colombia has one of the highest incidence rates for cervical cancer in the world (Table 4.4). Although study no. 26 showed the prevalence of HSV-2 antibodies was similar among patients and controls in Colombia, it is of interest that study no. 27, employing an immunofluorescence technique, showed that patients had higher titres than controls. Among patients from Israel, a subsequent study (no. 29) showed a higher proportion of patients than controls to possess HSV-2 antibodies but this proportion was even lower (15.4%) than that observed in the New Zealand studies (31-35%). Within the studies in which a higher proportion of cervical carcinoma patients were found to possess HSV-2 antibodies, there was considerable variation in the porportion of patients who were considered to be HSV-2 positive, e.g. 15% to 100%. This considerable variation in the prevalence of HSV-2 antibodies in the above sero-epidemiological studies precludes drawing definite conclusions regarding the aetiologic role of the virus in cervical carcinoma. The serologic assays used in these studies are only a crude measure of past infection by HSV-2 (Rawls et al, 1977),

RETROSPECTIVE STUDIES

Table 1.4 Studies on populations in N. AMERICA and the WEST INDIES which attempted to estimate the proportion of patients with cervical anaplasia and controls who possessed HSV-2 specific antibody.

COUNTRY, AREA, AND REFERENCE	PREDOMINANT RACE	TECHNIQUE	PROPORTION OF POSITIVES	
			CASES	CONTROLS
1. USA - Houston Rawls, Tompkins and Melnick (1969)	Negroid	Kinetic neutralization. Mean K values of 3.8 or greater to HSV-2 virus considered to represent the presence of HSV-2 specific antibody.	a) Dysplasias 24% b) Ca *in situ* 35% c) Inv. Ca. 72%	22%
2. USA - Baltimore Royston and Aurelian (1970a)	Negroid	Neutralization assay by plaque reduction using a mixture of HSV-1 & HSV-2 with distinct plaque morphology.	a) Dysplasias 95% b) Ca. *in situ* 100% c) Inv. Ca. 100%	44% 53% 67%
3. USA - Atlanta Nahmias *et al* (1970a)	Negroid	Microneutralization depending on CPE, expressed as a pN differences. Type-2 antibody considered to be present if pN type-1 - pN type-2 was < 0.5	Inv. Ca. 83%	35%
4. USA - Chicago Plummer and Masterson (1971)	Caucasian	Kinetic neutralization	Inv. Ca. 48%	18%

Table 1.4 (continued)

COUNTRY, AREA, AND REFERENCE	PREDOMINANT RACE	TECHNIQUE	PROPORTION OF POSITIVES	
			CASES	CONTROLS
5. USA - Houston	Negroid	Microneutralization test depending on cytopathic effect, expressed as II/I index.	80%	69%
6. USA - Houston	Caucasian		54%	25%
7. USA - Virginia	Caucasian		52%	23%
Rawls, Adam and Melnick (1972)				
8. West Indies - Barbados	Negroid	Complement fixation test. Type specific nature of the test is doubtful.	97%	76.6%
Ory et al (1973)				

Table 1.4 (cont'd) Studies on populations in EUROPE which attempted to estimate the proportion of patients with cervical anaplasia and controls who possessed HSV-2 specific antibody.

COUNTRY, AREA, AND REFERENCE	PREDOMINANT RACE	TECHNIQUE	PROPORTION OF POSITIVES	
			CASES	CONTROLS
9. England - Birmingham Skinner et al (1971)	Mainly Caucasian	Neutralization assay employing a mixture of HSV-1 & HSV-2 virus which were easily distinguished by plaque morphology.	a) Patients with dysplasia, Ca in situ and Inv. Ca had higher mean K values to the type-2 virus than controls. b) Patients and controls did not differ in mean K values to type-1 virus.	
10. England - Birmingham	Mainly Caucasian 4.6% coloured	Complement fixation test employing 'general virus antigen' and type-1 and type-2 specific antigen prepared by absorption of general antigen with heterotypic antibody	Dysplasia 33% Inv. Ca. 32%	12%
11. Ireland - Galway Skinner et al (1977)			Dysplasia 55% Inv. Ca. 53%	17%
12. Denmark - Copenhagen Vestergaard et al (1972)	Caucasian	Plaque reduction assay.	85%	47%
13. Belgium - Brussels Sprecher-Goldberger et al (1970)	Caucasian	Kinetic neutralization II/I ratio.	83%	29%

Table 1.4 (cont'd)

COUNTRY, AREA, AND REFERENCE	PREDOMINANT RACE	TECHNIQUE	PROPORTION OF POSITIVES	
			CASES	CONTROLS
14. Czechoslovakia - Prague Janda *et al* (1973)	Caucasian	Neutralization test using HSV-1 and HSV-2 viruses separately. II/I index used.	Dysplasia 50%	17%
			Ca *in situ* 48%	18%
			Inv. Ca 50%	21%
15. Hungary - Budapest Pasca *et al* (1975)	Caucasian	Indirect immunofluorescent test on acetone fixed HSV-1 and HSV-2 infected cells. HSV-2 antibody assumed to be present if the titre on HSV-2 infected cells were equal to or higher than the titre to HSV-1 infected cells	Ca *in situ* 68%	9%
			Inv. Ca 51%	
		Neutralization test employing HSV-1 and HSV-2 cells. Serum was considered to contain HSV-2 specific antibody if the serum had a neutralization endpoint to HSV-2 cells equal to or above that to HSV-1 infected cells.	Ca *in situ* 56%	9%
			Inv. Ca. 50%	
16. Finland - Turku Peltonen (1975)	Caucasian	Kinetic neutralization assay. II/I index.	Dysplasia 38%	
			Ca *in situ* 35%	18%
			Inv. Ca 47%	
17. Sweden - Stockholm Christenson & Espmark *(1976)*	Caucasian	Mixed haemadsorption test employing HSV-2 infected cells.	62%	23%

Table 1.4 (cont'd) Studies on populations in ASIA which attempted to estimate the proportion of patients with cervical anaplasia and controls who possessed HSV-2 specific antibodies.

COUNTRY, AREA, AND REFERENCE	PREDOMINANT RACE	TECHNIQUE	PROPORTION OF POSITIVES	
			CASES	CONTROLS
18. Formosa - Taiwan Rawls, Adam and Melnick (1972)	Mongoloid	Microneutralization test depending on cytopathic effect.	48%	50%
19. Formosa - Taiwan Kao et al (1974)	Mongoloid	Indirect immunofluorescent test on HSV-2 infected acetone fixed cells.	79%	63%
		Microneutralization test using HSV-2 virus.	100%	97%
		HSV-1 infected cells and HSV-1 virus not done.		
20. Japan - Kawana et al (1974)	Mongoloid	Kinetic neutralization - HSV-2 specific antibody titre considered to be SpK2 = K2-0.2 x K1	75%	71%
21. India - New Delhi Seth et al (1978)	Asian	Indirect haemagglutination by II/I index.	64%	36%
		Inhibition of indirect haem-agglutination IHAT	32%	27%

Table 1.4 (cont'd) Studies on populations in AFRICA which attempted to estimate the proportions of patients with cervical anaplasia and controls who possessed HSV-2 specific antibodies.

COUNTRY, AREA, AND REFERENCE	PREDOMINANT RACE	TECHNIQUE	PROPORTION OF POSTIVES	
			CASES	CONTROLS
22. Nigeria - Ibaden Adelusi et al (1975)	Negroid	Indirect immunofluorescent assay on acetone fixed HSV-2 infected cells.	70% 70% of patients had titres higher than 640 - only 11% of controls had the same.	11%
23. Uganda - Kampala Adam et al (1972a)	Negroid	Microneutralization test depending on cytopathic effect expressed as II/I index.		
	a) Ganda women		90%	71%
	b) non-Ganda women		67%	74%
24. Uganda Rawls, Adam and Melnick (1972)	Negroid	Microneutralization test depending on cytopathic effect, expressed as II/I index.	34%	69%
25. South Africa - Johannesburg Freedman et al (1974)	Negroid	Neutralization using HSV-1 and HSV-2 virus.	Patients had higher titres of HSV-2 antibody than controls. Antibody prevalence not given.	

Table 1.4 (cont'd) Studies on population in S. AMERICA, ISRAEL and NEW ZEALAND which attempted to estimate the proportion of patients with cervical anaplasia and controls who possessed HSV-2 specific antibodies.

COUNTRY, AREA, AND REFERENCE	PREDOMINANT RACE	TECHNIQUE	PROPORTION OF POSITIVES	
			CASES	CONTROLS
26. Colombia - Rawls, Adam & Melnick (1972)	Mestizo	Microneutralization test depending on cytopathic effect, expressed as II/I index.	36%	35%
27. Colombia Munoz et al (1975)	Mestizo	Indirect immunofluorescence test on HSV-2 and HSV-1 infected acetone fixed cells.	100% Patients had higher titres than controls.	100%
28. Israel Pridan & Lilienfeld (1971)	Caucasian	Kinetic neutralization II/I index. Tested by Rawls et al in Houston, USA.	35%	51%
29. Israel Menczer (1975)	Caucasian	Plaque reduction assay employing HSV-1 and HSV-2 infected cells. Considered to possess HSV-2 specific antibody if the HSV-2 titre was equal or greater than the HSV-1 titre.	15.4%	5.3%
30. New Zealand - Rawls, Adam & Melnick (1972)	Caucasian	Microneutralization test depending on cytopathic effect - expressed as II/I index.	35%	24%
31. New Zealand Rawls et al (1970b)	Caucasian	Microneutralization test depending on cytopathic effect, expressed as II/I index.	Ca in situ 32% Inv. Ca 31%	23%

for they detect antibodies to type-common as well as type-specific antigens. It has been shown by McClung et al (1976) employing a ^{51}Cr release assay, by Skinner et al (1974) employing immunoprecipitation, and by Vestergaard (Personal communication) by crossed immune electrophoresis that the predominant antibody activities following any HSV-1 or HSV-2 infection are to type-common antigens. McClung et al (1976) showed that 80-90% of antibody activity was directed towards type-ccmmon antigens and only 10-20% to type-specific antigens. He also observed that the type-common antigens of the two viruses were not identical because serum raised against the HSV-2 virus bound at similar rates to cells infected with either HSV-1 or HSV-2, in contrast to serum raised against HSV-1 which reacted more slowly with cells infected with HSV-2 than to cells infected with HSV-1. Therefore, since a proportion of the antigens on the cell surface are similar to those on the viral envelope (Lesso et al, 1970) it may be expected that whereas a serum with anti-HSV-2 antibody will have equal avidity for HSV-1 and HSV-2, a serum with anti-HSV-1 will have greater avidity for HSV-1, i.e. this could result in the inability to recognise low titre anti-HSV-2 antibody by kinetic neutralization assays which are a measure of both antibody avidity and antibody quantity. Smith et al (1972a) found by ^{51}Cr release assay that production of HSV-2 specific antibody was greater among patients without prior HSV-1 infection. Skinner et al (1976) found neutralization test capable of predicting with 95% confidence limit the immunising virus type only in seven of 40 antisera of known antitype. It is

expected that there would be variation in levels of exposure to the two viruses in different socioeconomic settings and in different countries. This, in combination with the nature of the immune response described above to the two viruses, may account for the wide variation observed in these case controlled studies. Attempts to overcome the obvious disadvantages of neutralization tests has led to the development of a complement-fixation assay, employing type-specific antigen (Skinner et al, 1976), a solid phase radioimmunoassay (RIA) based upon absorption of sera separately with uninfected cells, HSV-1 infected cells and HSV-2 infected cells, following which the absorbed sera are tested for residual antibody (Forghani et al, 1975), and an enzyme linked immunoabsorbent assay (ELISA) employing HSV-1 and HSV-2 specific antigens (Vestergaard et al, 1977b; Grauballe and Vestergaard, 1977). The ELISA technique may be as sensitive as RIA (Voller et al, 1976).

SEVEN CRITERIA WHICH IF FULFILLED WOULD PROVIDE EVIDENCE THAT HSV-2 WAS AETIOLOGICALLY RELATED TO CERVICAL CARCINOMA

The earlier prospective and retrospective studies described were very suggestive that HSV-2 was associated with cervical carcinoma. However they yielded no information as to whether the relationship was of a causal nature. Rawls *et al* (1977) have proposed ciriteria which if fulfilled would provide evidence that a particular virus was the causative agent of a particular malignancy. In this thesis these criteria have been modified. The evidence linking HSV-2 to cervical carcinoma has been weighed against these modified criteria in order to assess to what extent they have been fulfilled so far.

1. The epidemiological characteristics of women with cervical carcinoma and genital herpes should be similar.

2. Women with cervical carcinoma would be expected to have a higher frequency or higher titres of antibody to virus specified antigens and assays of cell mediated immunity should show positive or negative correlation.

3. The virus should be shown to cause cervical lesions which should occur well before the onset of neoplastic change and a higher incidence of the tumor should be observed in these women.

4. Similar viruses of the herpes group should cause naturally occurring malignancies in other animals.

5. The virus should be able to induce tumors in its natural host or in similar species and be able to transform cells in culture. The transformed cells in turn should be similar to tumor cells cultured in vitro and be able to induce tumors similar to naturally occurring ones.

6. There should be biological evidence linking one to the other, i.e. it should be possible to detect viral products (viral antigen), enzymes and nucleic acid in the tumor.

7. Individuals protected from developing genital herpes infection should demonstrate a lower incidence of cervical anaplasia in later years.

CRITERION NUMBER 1

THE EPIDEMIOLOGICAL CHARACTERISTICS OF WOMEN WITH CERVICAL CARCINOMA AND GENITAL HERPES SHOULD BE SIMILAR

Evidence for the venereal mode of transmission of the virus is reviewed on page 3 and from the description of the epidemiological features of the malignancy (pages 4-7) it is evident that the epidemiological features are similar. One epidemiological feature, however, requires further resolution, i.e. the mechanism whereby early age of first coitus acts as a high risk factor. Two possibilities have been suggested:

i) The importance of biological factors during early adolescence

From the colposcopic and laboratory studies of the cervices of 14,000 females of all ages (Coppleson and Reid, 1967), adolescence and first pregnancy were identified as periods of active squamous metaplasia in the cervix (page 73). Their studies and those of Singer (page 73) on the differences in the transformation zone of sexually active adolescents and virgin adolescents emphasise the importance of biological factors during a period of active metaplasia, which makes the cervical epithelium more susceptible to the action of a sexually transmitted agent and results in the development of an abnormal transformation zone. Since an abnormal transformation zone is suspected of harbouring cells with neoplastic potential, this provides one explanation for the importance of early age of first coitus as a high risk factor.

ii) The importance of behavioural factors

The second suggestion has come from Rawls et al (1976) that women who begin sexual activity at an early age also have multiple sexual partners.

CRITERION NUMBER 2

WOMEN WITH CERVICAL CARCINOMA WOULD BE EXPECTED TO HAVE A HIGHER FREQUENCY OR HIGHER TITRE OF ANTIBODY TO VIRUS SPECIFIED ANTIGENS, AND ASSAYS OF CELL MEDIATED IMMUNITY SHOULD SHOW POSITIVE OR NEGATIVE CORRELATION

Following the earlier case control studies employing mainly neutralization assays, later studies examined antibodies to antigens other than those involved in neutralization.

Antibodies to AG-4

Aurelian et al (1973) harvested an antigen (AG-4) four hours after infecting cells at low multiplicity (0.5 pfu/cell) and used this to detect antibody by means of a microcomplement fixation test. Antibodies to AG-4 were detected in the sera of 35% of patients with dysplasia, 65% of patients with carcinoma in situ and 35% of patients with invasive carcinoma, but only in 12% of controls. These antibodies belonged to the IgM class, and disappeared with successful treatment and reappeared with recurrence of the cancer. It was claimed that these antibodies were not associated with malignancies at other sites. The antigen was characterised as a molecule of molecular weight 161,000 which co-migrated on polyacrylamide gel with a minor virion protein (Aurelian and Strnad, 1976). The antigen was present in the cytoplasm as well as on the cell surface of infected cells. The reactivity of 8 of 10 tumor tissue biopsy extracts (Aurelian et al, 1977) with AG-4 positive sera constituted the evidence that AG-4 was present in cervical cancer cells. Kawana and his colleagues (1976) detected a lower prevalence of AG-4 antibodies among Japanese women with cervical cancer and suggested that this might be explained by the frequent isolation of HSV-1 from cases of genital herpes in Japan and that HSV-1 too may be related to cervical carcinoma.

Notter and Docherty (1976a) confirmed the high prevalence of AG-4 antibodies (78%) among cervical carcinoma patients but in contrast with earlier reports, detected the antibodies in one-third of patients with extra-genital non-squamous malignancies and two-thirds of patients with non-genital squamous malignancies.

Antibodies to VP-134

VP-134 is a non-virion polypeptide described by Anzai et al (1975) which is synthesised early in the infectious cycle and has a molecular weight of 134,000. These workers demonstrated quantitatively employing a method of immune precipitation that sera from cervical cancer patients precipitated more VP-134 than sera from patients with breast carcinoma or from women who did not have cancer.

Antibodies to NVA-TAA (non-virion antigen-tumor associated antigen)

This is a soluble membrane antigen which was first isolated from the supernate of sonicated vaginal, vulval, cervical and lip cancer cells, which cross-reacted with an antigen on cells infected by herpes simplex virus (Hollinshead et al, 1972; Hollinshead and Tarro, 1973). A high proportion of patients with a variety of squamous cell tumors, including patients with cervical carcinoma possessed antibody to these antigens (Hollinshead and Tarro, 1973). This antigen is discussed in detail on page 282.

Evidence for a cross-reacting antigen between HSV-transformed cells and squamous carcinoma cells

(Notter and Docherty, 1976b)

Antigen prepared from HSV-1 and HSV-2 transformed cells were tested in a microcomplement fixation assay with sera from patients with squamous carcinoma. Ninety-four per cent of the sera reacted with the HSV-1 transformed cell antigen and 84% with HSV-2 transformed cell antigen. The proportion of sera from patients with other non-squamous malignancies which reacted with these antigens was similar to that among

controls with no malignancy. When sera from all individuals (normal and cancer) were tested against similar antigen prepared from CMV transformed cells, no significant reaction pattern was observed.

Cytotoxic antibodies

Women with progressing cervical lesions were found to have lower complement-dependent cytotoxic antibody titres to HSV-2 infected cells and HSV-2 transformed cells than women with regressing lesions (Thiry et al, 1974)

Christenson (1977) found that patients in the terminal stages of cervical carcinoma had higher cytotoxic antibody titres to cell lines derived from squamous cell tumors of the cervix than to HSV-2 infected cells.

The titres of cytotoxic antibody to HSV-2 infected cells thus appear to be inversely related to the severity of disease. These results are similar to the prevalence in these patients of antibody to HSV-2 infected cells, as determined by the mixed haemadsorption assay. Christenson and Espmark (1976) detected decreasing prevalence of these antibodies with increasing stage of disease.

Cell-mediated immunity (CMI) to HSV-2 antigen in patients with cervical carcinoma

Sprecher-Goldberger et al (1975) detected a non-specific cell mediated immunosuppresion in women with cervical carcinoma when compared with control women as judged by phytohaemagglutinin (PHA) stimulation of the whole white cell fraction from blood samples. Women who had been treated had indices of stimulation which were similar to those of controls.

In contrast with the results with PHA stimulation, indices of stimulation by HSV-2 and by HSV-2 transformed cells were higher in cervical carcinoma patients than controls. Here too stimulation indices of treated patients were similar to those of controls.

Although the results of seroepidemiological studies and the studies on cell mediated immunity are suggestive of an aetiological role for the virus, the association may also to a large extent be explained if a) one postulates that HSV-2 is a co-variable of the real carcinogen, or b) that the virus is a secondary invader of tumor tissue which favours its replication. While studies up till now have left the former possibility unanswered, evidence is available which argues against the latter possibility (see Criterion Number 3 below). The presence of a cross-reacting antigen between squamous carcinoma cells and HSV-2 infected and transformed cells could also confuse the results of both antibody assays and assays of cell mediated immunity, e.g. it may explain higher titres of antibody to HSV-2 infected cells among cancer patients compared to controls, as well as increasing titres of antibody with increasing stage of disease, if the particular assay believed to be HSV-specific is a test of antibody to this cross-reacting antigen as well.

CRITERION NUMBER 3

THE VIRUS SHOULD BE A PROVEN CAUSE OF CERVICAL LESIONS, WHICH SHOULD OCCUR WELL BEFORE THE ONSET OF NEOPLASTIC CHANGES AND A HIGHER INCIDENCE OF THE TUMOR SHOULD BE OBSERVED IN THESE WOMEN

The cervix as a frequent site of infection in genital herpes is well-documented (page 3). Naib et al (1969) reported the mean age of women with genital herpes, dysplasia, carcinoma in situ and invasive carcinoma to be 20, 25, 31

and 48 respectively. This argues against the possibility that the virus is a secondary invader of tumor tissue and also provides evidence that cervical lesions occur before the onset of neoplastic change. Further evidence for this is seen in a study by Catalano and Johnson (1971) who demonstrated HSV-2 specific antibody in the sera of five of 14 women who developed carcinoma <u>in situ</u> one to eight years after that particular sample of serum had been collected. Rawls and Gardner (1972) demonstrated that 80% of women with normal cervical cytology were infected when exposed to men with penile herpes. Since the infectivity rate of normal cervical epithelium is high it appears unnecessary to postulate previous anaplastic change to explain the high prevalence of HSV-2 antibody in women with carcinoma of the cervix. However, the possibility that (in a woman harbouring the latent virus) the neoplastic epithelium is more susceptible to recurrent attacks (clinical or subclinical) has not been explored. This possibility could also provide explanations for higher titres as well as fluctuating antibody with tumor progression, treatment and recurrence. As far as is known and suprisingly, there is no study which has examined the frequency of isolation of virus from tumor-bearing cervices in contrast with normal cervices.

<u>CRITERION NUMBER 4</u>

<u>SIMILAR VIRUSES OF THE HERPES GROUP SHOULD CAUSE NATURALLY OCCURRING MALIGNANCIES IN OTHER ANIMALS</u>

There is no malignancy in animals which is analogous to squamous carcinoma of the cervix. However, other members

of the Herpes group of viruses can induce malignancies in animals and the Epstein-Barr virus has been strongly linked to two human malignancies - Burkitt's lymphoma and naso-pharyngeal carcinoma.

Renal Adenocarcinoma of Frogs

The Lucké renal adenocarcinoma is histologically similar to the equivalent human tumor and occurs naturally in Rana pipiens, the leopard grog (Lucké, 1952). It exists in two states which are temperature-dependent. In winter tumors contain herpes virus-like particles whereas in the summer they do not. However, virus specific m-RNA has been detected in summer tumors (Collard et al, 1973a) explants from which develop virus nucleic acid and virus antigen and infectious particles when incubated in vitro at 7.5°C (Breidenbach et al, 1971). These findings indicate that although virus genetic information necessary for production of structural proteins is temperature-dependent, information necessary for cell transformation continues independently of temperature.

Marek's Disease of Chickens

This is a malignant lymphoma-leukaemia of T-lymphocytes which is developed by chickens infected with Marek's disease virus (MDV) shortly after they are hatched. It is prevented by vaccination of day-old chicks (Jose, 1978). There is evidence to suggest that the MDV may activate endogenous C-type RNA leukaemia virus but it it not known if this RNA virus is essential for the development of the disease (Frankel and Groupe, 1971).

Lymphoma of Guinea Pigs

The guinea pig herpes virus was isolated from leukaemic lymphoblasts and normal lymphocytes from a strain of guinea pigs with a high incidence of spontaneous leukaemia (Hsiung et al, 1971). An RNA virus has been found to be associated with these leulaemic cells and neoplastic disease could be induced only in guinea pigs inoculated with both viruses (Hsiung and McTighie, 1976).

Malignant Lymphoma of Monkeys

Herpes virus siamiri (HVS) and Herpes virus ateles (HVA) are not pathogenic in their own hosts, the South American squirrel monkey and the South American spider monkey respectively. However, both viruses induce a lymphoproliferative disease with leukaemia of the T-cell type in New World monkeys, e.g. cotton topped and white lipped marmosets and owl monkeys, and in New Zealand white rabbits (HVS). Virus particles and antigens have not been detected in malignant cells obtained directly from the tumors but were detectable in a small proportion (1-5%) of tumor cells when explanted into tissue culture. HVS and HVA genome has been demonstrated in the neoplastic lymphoblastic cells (reviewed in Jose, 1976).

Burkitt's Lymphoma (BL)

The concept of the viral aetiology for Burkitt lymphoma has gained strength on the following evidence.

i) The virus was first detected in the culture fluid of a cell line established from a Burkitt lymphoma (Epstein et al, 1964).

ii) It has *in vivo* oncogenic potential in non-human primates (Werner *et al*, 1972).

iii) BL patients have higher levels of antibody against a wide range of EBV determined antigens, e.g. EBV capsid antigen, early antigen, and membrane antigen (Epstein, 1978). Also viral antibodies vary with disease events, e.g. a fall in antibody titre to late membrane antigens have been noticed four to six months prior to recurrence (Klein, 1975).

iv) Every tumor cell has been found to carry virus DNA (Epstein, 1978b) and EBV determined nuclear antigen has often been detected in BL cells (zur Hausen, 1970).

Nasopharyngeal Carcinoma (NPC)

This is the commonest malignant tumor in Chinese males and the second commonest in Chinese females (Shanmugaratnam, 1967). The tumor arises in the post nasal space, and more viral genome has been found in the epithelial tumor cells than the lymphoid cells (Klein et al, 1974). Also, inoculation of tumor cells into athymic mice results only in the outgrowth of epithelial cells (Trumper et al, 1977) suggesting the epithelial cell to be the primary malignant cell. Genetic factors appear to play a role in the pathogenesis of NPC as an association has been shown between HLA types A_2-B Sin 2, BW 17-AW 19, and BW17-A blank, and the tumor (Simons et al, 1978). On the other hand although NPC has a marked geographic prevalence, no definite environmental co-factor has yet been identified as influencing its pathogenesis. However a study of Chinese immigrants from South-east Asia living in Australia and the United States has shown that environmental factors must play a part. The first generation of these immigrants had the same high incidence of NPC as the Chinese in China. A lower incidence of NPC was seen in Chinese descendents born in Australia and the USA which was nevertheless higher than that of the surrounding caucasian population (Epstein and Achong, 1977). Thus it would appear likely that EBV acts with some as yet unknown environmental co-factor, on a susceptible genetic constitution, to bring about the malignant change of NPC.

CRITERION NUMBER 5

THE VIRUS SHOULD BE ABLE TO INDUCE TUMORS IN ITS NATURAL HOST OR IN SIMILAR SPECIES AND BE ABLE TO TRANSFORM CELLS IN CULTURE. THE TRANSFORMED CELL IN TURN SHOULD BE SIMILAR TO TUMOR CELLS CULTURED IN VITRO AND BE ABLE TO INDUCE TUMORS SIMILAR TO NATURALLY OCCURRING ONES.

For obvious ethical reasons the ability of the virus to induce tumors in humans cannot be experimentally tested. The alternative of proving tumor induction in animals has been attempted but has remained a relatively ill developed field.

i) Experiments in Hamsters

The inoculation of newborn hamsters with HSV-1 and HSV-2 intravaginally with virus doses greater than 10^3 TCD50 resulted in nearly 100% mortality. About 75% survived with lower doses of live virus or UV-inactivated virus. Two to three per cent of HSV-2 infected, but not HSV-1 infected, animals developed undifferentiated sarcomas 5-28 months after inoculation (Nahmias et al, 1970b). Neither HSV-2 antibody nor viral antigen could be demonstrated in the serum or tumors of these animals. However, HSV-2 antigens were detected in two transplanted tumors (note similarity in this respect to malignant lymphoma in monkeys). These tumor explants were examined for C-type particles but none were detected.

ii) Experiments on Mice

Adenocarcinomas have been observed in a few mice following genital inoculation of formaldehyde treated HSV-2

(Wentz et al, 1975). Also a few cases of squamous carcinoma in mice which were inoculated genitally with non-inactivated HSV-2 while they were being given female hormones (Munoz, 1973). In the latter experiments the administration of hormones confused the interpretation of these results.

iii) Experiments on Cebus Monkeys

Nahmias et al (1971b) demonstrated that cebus monkeys inoculated vaginally with HSV-2 developed genital infection which was similar to that developed by women. Similarities were seen in the incubation period, clinical appearance of lesions, duration of virus recovery, and the development of serum neutralising antibodies. On reinfecting these monkeys 6-12 weeks following initial inoculation, 3 of 7 monkeys developed lesions despite having high neutralising antibody titres. However in this study or in the subsequent study conducted on cebus monkeys, it is not stated whether these animals once infected suffered from endogenous recurrent infection as women do. Similar attempts at infecting squirrel monkeys and rhesus monkeys were unsuccessful.

Palmer et al (1976) reported on an ongoing study investigating the oncogenic potential of HSV-2 in 301 female cebus monkeys. The virus was inoculated intraepithelially into the cervix every six months. Control animals received an inoculation of 10% salicylic acid in mineral oil which produced severe lesions which subsequently healed. Sixty-six virus recipients have so far been followed up over a period of 14 or more months up to the time of reporting. Thirteen (19.7%) developed mild-moderate dysplasia. These dysplastic lesions persisted up to 12-32 months. The results on the salicylic acid inoculated animals were not reported.

These results are encouraging from the point of view of establishing a suitable animal model for the study of cervical carcinoma. However no firm conclusions can be made as to the oncogenic potential of HSV-2 until the results of follow up studies show that the animals develop malignant lesions. Because in these experiments inoculation of virus was repeated every six months it is impossible to judge if dysplasia would have regressed with time. This is an important question among humans, i.e. whether the dysplastic lesions which have been observed in greater numbers among women with genital herpes are a transient feature. These experiments may have been designed better by inoculating some animals repeatedly and others only once.

Transformation of Cells

Transforming assays are usually carried out on non-permissive cells in which transforming functions are expressed but replication blocked. Since no cultured cell which is totally non-permissive to HSV-1 and HSV-2 is available, the earliest experiments on transformation by these two viruses employed viruses inactivated either by ultraviolet irradiation or a combination of visible light and neutral red (Studies 1-5, Table 1.5). By these methods viral replication was blocked but transforming activity was not. Other methods employed to evade productive infection of cells included growing the cells at non-permissive temperatures (Study 6, Table 1.5) and the use of temperature sensitive mutants (Studies 7, 8 and 9). Recently, mechanically sheared DNA has been used as the process of mechanical

Table 1.5 Transformation of cells by HSV-1 and HSV-2

REFERENCE AND VIRUS	CELL LINE	ONCOGENICITY	HERPES ANTIBODIES IN ANIMALS AND HERPES ANTIGENS IN TUMORS
1. Duff and Rapp, 1971 a and b. UV inactivated HSV-2.	Primary hamster embryo fibroblasts.	Induced fibrosarcomas in hamsters, 10-16 weeks after inoculation.	Low levels of neutralising antibody against HSV-1 and HSV-2 in the serum of tumor-bearing animals.
2. Rapp and Duff, 1973; Duff and Rapp, 1973. UV inactivated HSV-1.	Primary hamster embryo fibroblasts.	Adenocarcinoma-like tumors in 47% of newborn hamsters, in 3-11 weeks.	Ditto.
3. Kutinova et al, 1973. UV inactivated HSV-2.	Continuous line of hamster cells which had oncogenic potential.	Cells became more oncogenic - only 1/10th the previous dose being sufficient for tumor formation.	Not done.
4. Boyd and Orme, 1975. UV inactivated HSV-2.	Non-oncogenic 3T3-like line of Balb/C mouse cells.	75-80% of mice infected with 10^6 cells developed fibroblasts.	Tumor-bearing animals developed neutralising antibody HSV-2 antigen in cytoplasm of tumor cells by immune fluorescence.
5. Rapp et al, 1973. Visible light and neutral red inactivated HSV.	Hamster embryo fibroblasts.	Not tested	Cytoplasmic fluorescence in transformed cells tested against anti-HSV hamster serum.
6. Munk and Darai, 1973; Darai et al, 1975. Uninactivated HSV-2 8 days at 42°C.	Human embryo lung cells.	Not inoculated into animals. Cell line maintained for over 65 passages. When subsequently incubated at 37°C, unable to undergo replication although viral DNA was present in the cells.	Viral antigens demonstrated in the cytoplasm of the cells.
7. Macnab, 1974 and 1975. ts mutants of HSV-1.	Rat embryo cell line.	Oncogenic in rats.	Viral antigens detected in the cytoplasm and the surface of transformed cells.

Table 1.5 (cont'd)

REFERENCE AND VIRUS	CELL LINE	ONCOGENICITY	HERPES ANTIBODIES IN ANIMALS AND HERPES ANTIGENS IN TUMORS
8. Macnab, 1979. ts mutants of HSV-2 and UV irradiated HSV-2.	Rat embryo cells	Fibrosarcoma in immunosuppressed rats. Latent period as long as 2 years in some cases.	Perinuclear and cytoplasmic fluorescence in the cells of tumors which developed as long as 36 weeks following inoculation.
9. Takahashi and Yamanishi, 1974. HSV-2 ts mutants.	Hamster embryo cells and human.		Viral antigens detected in the cytoplasm of 5-10% of transformed cells.
10. Wilkie et al, 1974. Mechanically sheared HSV-1 DNA.	Primary rat embryo cells.		
11. Jariwalla et al, 1978. Calcium phosphate co-precipitated HSV-2 DNA.	Syrian hamster embryo.	Produced fibrosarcoma in 100% of newborn hamsters.	HSV type common antigen (Ag-e) demonstrated in the transformed cells and tumor cells.

shearing eliminates the ability of viral DNA to replicate. (Studies 10 and 11). Virus specific RNA (Collard et al, 1973b) and 1-3 copies per cell of viral DNA sequences representing 8-32% of the genome (Frenkel et al, 1976) have been demonstrated in some of the HSV transformed cell lines but not in others (Davis and Kingsbury, 1976). Transformation experiments up to date have proved that the virus is capable under certain conditions of bestowing on cells properties that are associated with human malignant cells, i.e.

i) The ability to produce neoplasms in animals

ii) Of being "immortal"

iii) Of being able to grow in soft agar

iv) Of having a greater efficiency of cloning than non-transformed cells.

This property of the virus provides a good foundation for the hypothesis that it may be a cause of human malignancy. It also provides a model for virus genome and cell genome interaction in a malignant cell. In addition the production of tumors in animals by the inoculation of transformed cells may provide information about host factors which are important in tumor development.

However few transformation experiments have been performed on human cells (Study 6) and these were not tested for their oncogenic potential in animals. The physical state of the viral DNA within the transformed cell too has not been established. The rat model (Study 8) is interesting as the latent period between inoculation of transformed cells and tumor development was as long as two years in some rats. Elucidation of the mechanism whereby oncogenic potential is

conferred on the rate embryo cell by the virus would be relevant to the possible mechanism (if be) in cervical carcinoma, which also often has a long latent period in development. This aspect of long latency in the rat model is being studied further by these workers.

CRITERION NUMBER 6

THERE SHOULD BE BIOLOGICAL EVIDENCE LINKING ONE TO THE OTHER, I.E. IT SHOULD BE POSSIBLE TO DETECT VIRAL PRODUCTS (VIRAL ANTIGEN) ENZYMES AND NUCLEIC ACID IN THE TUMOR

Viral antigens in cervical carcinoma cells

AG-4 (page 27) and NVA-TAA (page 28) are two HSV antigens which have been demonstrated in cervical carcinoma tissue and which have been subsequently characterised. So far, AG-4 has been reported as detected in eight tumor biopsies. NVA-TAA is not specific for cervical carcinoma and has been extracted from lip, vaginal and vulval squamous cancers as well.

Royston and Aurelian (1970b) demonstrated herpes simplex antigen in the exfoliated cervical cells of patients with cervical anaplasia by a direct fluorescent technique and an indirect fluorescent technique employing rabbit anti-serum raised against HSV-2. Exfoliated cells from 21 of 26 patients with dysplasia or carcinoma in situ and 7 of 10 patients with invasive carcinoma gave positive fluorescence with rabbit anti-HSV-2 serum in contrast with negative fluorescence observed in specimens of exfoliated cervical cells from 10 women with normal cervices.

Pacsa et al (1977) conducted similar experiments using an indirect fluorescent test. The study group consisted of a representative sample of women. Herpes antigen was detected in a significantly high proportion of women with cervical cancer (91.8%) (Table 1.6). Similar results were obtained by Adelusi et al (1976).

Table 1.6 Incidence of herpes simplex virus capsid antigens in exfoliated cervical cells of virgins and sexually active women. From Pacsa et al (1977).

GROUP	NO. TESTED	NO. OF POSITIVES	%
1. Virgins	106	0	0
2. Women with normal cervical epithelium	710	64	9.1
3. Women with non-malignant disorders	205	79	38.0
4. Patients with cervical dysplasia	62	35	56.4
5. Patients with invasive cervical carcinoma	49	45	91.8

No further work has been reported which has attempted to characterize these antigens. Although monospecific sera to a number of herpes polypeptides have been prepared in several laboratories, attempts have not been made to detect viral polypeptides in biopsied tumor tissue.

Viral Nucleic Acids in Human Tumors

Frenkel et al (1972) reported the detection of HSV-2 specific DNA and RNA in one cervical carcinoma. The segment

of DNA detected represented 39% of the HSV-2 genome. Although attempts were made by other workers to repeat these experiments (zur Hausen et al, 1974; Schulte-Holthausen, 1975; Pagano, 1975) they yielded no success.

CRITERION NUMBER 7

INDIVIDUALS PROTECTED FROM DEVELOPING GENITAL HERPES INFECTION SHOULD DEMONSTRATE A LOWER INCIDENCE OF CERVICAL NEOPLASIA IN LATER YEARS

This is a logical and an attractive idea. However conducting such experiments is fraught with difficulty. There is current speculation on the possible protection that previous HSV-1 infection may confer on the development of genital herpes. These are described on page 105 of the Introduction, and discussed further on page 318 of the Discussion.

Thus none of the criteria have so far been completely satisfied. The epidemiological features of genital herpes and cervical carcinoma are almost identical but the significance of the early onset of coitus in the pathogenesis of the malignancy in relation to the herpes hypothesis needs to be clarified.

Retrospective and seroepidemiologic studies and prospective studies strongly suggest that HSV-2 may be aetiologically related but the question of whether the virus is a co-variable of the real carcinogen has not been answered.

Experiments on cebus monkeys, although encouraging, are so far inconclusive as none of the monkeys have developed a pre-invasive or invasive lesion. It may provide a suitable experimental model for the study of co-factors, but as an experimental model is expensive.

The failure to demonstrate the herpes genome in tumor cells may reflect the insensitivity of currently available probes in detecting fractions of the genome. In adenovirus transformed cells as little as 4-5% of the genome is present in transformed cells (Pitot, 1978). However two techniques which have recently been developed may help to increase the sensitivity of molecular probes:

i) Restriction enzyme technology

ii) Transformation of cells with DNA fragments.

If a particular fragment of DNA is found to consistently bring about oncogenic transformation of cells, it would enable sensitive probes to be prepared from such fragments in order to identify similar genetic material in tumor cells. This technology is at present being rapidly developed.

Another approach would be to identify the part of the genome which codes for tumor associated antigens such as AG-4, VP134, and others demonstrated by immunofluorescence (pages 42&43)and to search for this part of the genome in tumor cells. A number of herpes polypeptides have been mapped on the herpes genome in Roizman's laboratory (page 61). Antigens such as AG-4 and VP134 require to be related to such polypeptides.

Since the epidemiological features of cervical carcinoma point to a venereally transmitted agent as the aetiologic agent, other such agents have to be considered (Table 1.7). This aspect of cervical carcinoma has remained a neglected field.

OTHER VENEREALLY TRANSMITTED AGENTS

Smegma

The increased incidence of carcinoma of the penis in uncircumcised males (Bleich, 1950) led to the consideration of smegma as a possible aetiologic agent. The evidence from experiments conducted in mice is contradictory (reviewed by Coppleson, 1969) and has failed to prove its mutagenic action. In addition, two studies showed no relationship between the occurrence of carcinoma of the cervix and the circumcision status of the husband (Aitken-Swan and Baird, 1965; Terris *et al*, 1972).

Spermatozoa

The possibility of spermatozoa being mutagenic was first suggested by Reid (1964). These studies investigated the role of sperm head DNA (Coppleson and Reid, 1967) as well as sperm head proteins (Reid *et al*, 1978). The development of the transformation zone of the cervical epithelium has been described on page 73 . When semen was cultured *in vitro* with biopsied mature squamous epithelium from the transformation zone or with cervical columnar epithelium, sperms never gained access to the cells of the tissue. In contrast, when

Table 1.7 Venereally transmitted agents of possible importance in cervical carcinoma

AGENT	OBSERVED WITHIN THE CELLS OF THE CERVICAL EPITHELIUM	CAUSES CERVICAL LESIONS	RECOVERY FROM SEMEN
Smegma			
Spermatozoa	Phagocytosed by epithelial cells in metaplastic phase	No	+
Trichomonas vaginalis	No	Yes	-
Neisseria gonorrhoea	No	Yes	-
Treponema pallidum	No	Yes 40% of cases	-
Mycoplasma	Yes	Yes	+
Chlamydia	Yes	Yes	-
Cytomegalovirus	Yes	Yes	+
Human papilloma	Yes	Less frequently than vulva	-

semen was cultured with biopsy material removed from the cervix during a phase of metaplasia, e.g. immediately following first pregnancy, the cells at times phaged sperms. In vivo studies where biopsy material was taken from immature metaplastic epithelium of the cervix 7-9 hours after coitus confirmed these in vitro studies. Similar experiments conducted on mouse uterus employing tritiated thymidine labelled mouse sperm demonstrated labelled sperm within the nucleus of dividing cells and chemical analysis of such DNA molecules demonstrated a change in chemistry, showing a graph peak in the same area as the donor sperm. These experiments demonstrated one possible manner in which mutational change could be brought about in the cervical epithelium and more important, emphasized the possibility that the target tissue had its own periods of susceptibility, i.e. during its phases of metaplasia. This hypothesis offers one possible explanation for the decreased incidence of cervical carcinoma amongst Jewish women. Jewish religious doctrines forbid coitus during and immediately following menses, during certain antepartum and postpartum periods and during any vaginal haemorrhagic discharge.

Studies on the two basic sperm head proteins, histone and protamine, showed wide variation amongst different males and a ratio between the two proteins which correlated with social class. The lower the social class, the greater the proportion of protamine. This was so for specimens of seminal fluid collected from Australia as well as England. These workers (Reid et al, 1978) suggest that sperm head protamine may thus have a role in squamous carcinoma of the

cervix which is also commoner in the lower social classes.

Thus, according to the spermatozoan theory, the mutagenic event is limited by properties of sperm head proteins as well as properties of the cervical epithelium (limited to periods of metaplasia).

Trichomonas vaginalis

Trichomonas is sexually transmitted and has been shown to be associated with abnormal cervical cytology (Bechtold et al, 1952; Meisels, 1969) and with invasive carcinoma (Berggren, 1969). However, it was demonstrated that with treatment of the infection early neoplastic changes regressed (Bertini and Hornstein, 1970). These lesions therefore may not be truly precancerous. Also, there is no evidence to show that these organisms can enter cells of the genital tract.

Neisseria gonorrhoea

It has been suggested by Beilby et al (1968) that Neisseria gonorrhoea may activate latent cervical herpes in a manner similar to the reactivation of oral herpes by acute respiratory infection. In their study, eight of 209 women attending a venereal disease clinic had cytological and virological evidence of herpes virus infection of the cervix although clinical appearance of the lesion did not suggest a diagnosis of herpes. Fifty-four of the 209 women had gonococcal infection detected by culture and microscopy. Seven of the eight women from whom herpes virus was isolated were

among these 54 women infected with N. gonorrhoea in contrast with one patient from whom herpes virus was isolated among 155 women without gonococcal infection. In a study in a Seattle venereal disease clinic (Alexander, 1973) N. gonorrhoea infection was also found to be associated with both HSV-2 and chlamydial infection.

Treponema pallidum

Syphilis was the first venereal disease to be associated with cervical carcinoma (Levin et al, 1942). However more recent studies in the USA have failed to confirm this association (Wynder et al, 1954). This is probably a reflection of two factors:

a) the recent change in the epidemiology of venereal disease in the USA with declining incidence of syphilis, and

b) the possibility that the early association noticed was only a marker of greater sexual promiscuity in cervical carcinoma patients. A more recent study in Taiwan (Alexander, 1973) showed 25% seropositivity to Treponoma pallidum among cervical carcinoma patients in comparison to 10% seropositivity among matched controls. It is noteworthy that two studies in Taiwan failed to demonstrate an association between HSV-2 and cervical carcinoma (Table 1.4). Cervical involvement is seen in approximately 40% of women with syphilis (Slavin, 1976). However, the parasite is not usually seen within the epithelial cells.

Mycoplasma

The T strains of Mycoplasma and Mycoplasma hominis have

been more frequently isolated in populations attending venereal disease clinics (Gregory et al, 1970; Holt et al, 1967; Gutman et al, 1972), among prison populations (Ford and Du Vernet, 1963), and promiscuous persons (Solomon et al, 1970). Recovery of Mycoplasma from seminal fluid in males correlated with recovery from the cervical secretions of their partners (Gnarpe and Friberg, 1972). Mycoplasma has been isolated from cases of chronic inflammatory disease of the cervix (Dunlop et al, 1974). In the study done by Gregory et al (1970) a correlation was observed between mycoplasma infection and dysplasia. However no serologic studies have been reported in which the prevalence of mycoplasma antibodies in patients with cervical carcinoma and controls were examined.

Chlamydiae

Schachter et al (1974) made a study of chlamydial infection in women with dysplasia (Table 1.8). There was a higher isolation rate from these women in comparison with women attending a cancer screening clinic. The highest isolation rate was from women seen at a women's clinic (which included women with inflammatory disease of the genital tract). The proportion of patients with dysplasia who had complement fixing chlamydial antibody titres of greater than 1:16 was higher than that seen in many women attending the cancer screening clinic (Table 1.8A), the venereal disease clinic or women with gonorrhoea (Table 1.8B). A significantly higher proportion of women with dysplasia had immunofluorescent antibody titres of over 1:64, compared to women attending

Table 1.8

A. Study by Schachter et al (1974) on chlamydial (TRIC) infection in patients with dysplasia

PARAMETER OF COMPARISON	127 WOMEN FROM CANCER SCREENING CLINIC	90 WOMEN FROM A WOMEN'S CLINIC*	176 WOMEN FRO A DYSPLASIA CLINIC
TRIC isolation	0.3%	7.8%	4.1%
Herpes isolation	1.0%	1.0%	1.0%
TRIC complement fixing antibody of titre >1:16	11.0%	21.0%	21.0%
TRIC fluorescent antibody of titre >1:8	67.3%	63.3%	77.6%
TRIC fluorescent antibody of titre >1:64	$\frac{16}{72}$ (22%)	$\frac{34}{74}$ (45%)	$\frac{32}{72}$ (88%)

* included women with inflammatory disorders of the genital tract

B. Chlamydial (TRIC) complement fixing antibodies in selected populations

	NUMBER TESTED	PERCENTAGE POSITIVE
Normal premarital women	47	2.1%
Pregnant women	297	5.0%
Women from venereal diseases clinic	221	12.2%
Women with gonorrhoea	116	14.6%
Prostitutes	30	30.0%
Women with proven TRIC agent infection	40	57.5%

the cancer screening clinic. Representative antigen from Trachoma inclusion conjunctivitis (TRIC) immune types A, B, C and D and F were used in these tests and most sera had broad reactivity to most or all of the antigens. Although this study does not demonstrate a specific association between TRIC agents and the dysplasia process, it does provide sufficient evidence for implicating it as a possible agent or co-agent - more so because it is an intracellular parasite. In a study conducted by Gutman et al (1972) among veneral disease clinic patients, chlamydiae were isolated from 26% of 100 cases of cervicitis in contrast to 11% of controls.

Cytomegalovirus

The results obtained in studies assessing the association between cytomegalovirus and carcinoma of the cervix has been contradictory. Fucillo et al (1971) examined the sera of patients in the USA who later developed carcinoma in situ (Table 1.9, Group a), sera of patients with concurrent carcinoma in situ (Group b) and sera of patients after they had had cone biopsy for carcinoma in situ (Group c). Both the prevalence of CMV antibodies and the titres in patients compared with controls indicated that CMV was not a causative agent or a secondary invader of neoplastic tissue. Both the prevalence of CMV antibodies and their titres were lower in patients who had had cone biopsy suggesting the cervical epithelium as the site of infection responsible for the maintenance of antibody levels.

In a study done by Munoz et al (1975) (Table 1.9) on

Table 1.9 Studies examining the association of cytomegalovirus (CMV) and cervical anaplasia

REFERENCE AND TECHNIQUE	STUDY GROUP	PROPORTION HAVING ANTIBODIES PATIENTS	CONTROLS	TITRES (LOG_2)	P
Fucillo et al (1971) Indirect haemagglutination	a) Serum from 14 patients prior to developing CIS.	71.0%		4.21	0.2
	a') 28 matched controls.		51%	3.57	
	b) Serum from 30 patients with CIS at time lesion was present	63.0%		4.6	0.2
	b') 60 matched controls.		71%	4.18	
	c) Serum from 11 patients after conisation for CIS	37.5%		2.75	0.03
	c') 22 matched controls		69%	4.03	
Munoz et al (1975) by Indirect immunofluorescence	22 patients with CIS and 35 with invasive carcinoma	100%			
1) VCA of CMV	104 matched controls		100%		
2) EA of CMV	50 patients with CIS	36%			
	99 controls		19%		
Vestergaard et al (1972) Complement fixation	135 patients with cervical carcinoma	80%			
	115 controls		65%		

CIS - carcinoma in situ

CMV - cytomegalovirus

VCA - virus capsid antigen

EA - early antigen

invasive carcinoma patients and controls in Colombia, 100% of patients and controls had antibodies to virus capsid antigen of CMV. However, higher frequency of antibodies to early antigens of CMV was observed in patients compared with controls. This may suggest a higher prevalence of active CMV infection in these patients.

In a study done by Vestergaard et al (1972) a higher proportion of cervical carcinoma patients were found to possess CMV complement fixing antibodies and in significantly higher titres.

The conflicting results in these studies may be attributed to the varying techniques employed in each study. It has been shown that the complement fixation test for CMV is less sensitive than the immunofluorescent test (Tsiantos et al, 1974). Further studies on antibodies to the early antigens of CMV infected cells have not been conducted. Although virus specific surface antigens have been demonstrated on the surface of CMV infected as well as CMV transformed cells (Albrecht and Rapp, 1973; Langenhuysen et al, 1970; Lausch et al, 1974) antibody to these antigens have not been studied in women with cervical anaplasia. It is a virus which deserves further investigation as a possible cervical carcinogen. It has been isolated from the uterine cervix (Goldman et al, 1969, collaborative study, 1970) more frequently than HSV-2 (Jordan et al, 1973) and more frequently in late pregnancy and the post partum period than in early pregnancy (Stagno et al, 1975). (During the post partum period after the first pregnancy there is active metaplasia

of the cervical epithelium, page 73). It has been demonstrated in the semen of asymptomatic carriers for over one year (Lang et al, 1974). Transformation of hamster embryo fibroblasts has been demonstrated after exposure to UV inactivated CMV (Albrecht and Rapp, 1973) and of human embryo lung fibroblasts persistently infected with a genital isolate of CMV (Gedder et al, 1976). It is likely that CMV is venereally transmitted as it is a labile virus, it is recovered from semen, and because there is a high isolation rate from the genital tracts of women attending venereal disease clinics (Jordan et al, 1973; Wentworth et al, 1973; Willmott, 1975).

Although CMV commonly infects the female genital tract, CMV infected cells are seen rarely in cervical smears. One possible explanation for this is seen in the results of in vitro studies conducted by Vesterinen et al (1975) into the cytopathogenicity of CMV for ecto- and endocervical epithelium. The endocervical cells were clearly more conducive to viral growth than the ectocervical cells. Endocervical cells are rarely seen in a cervical smear. Occasional CMV inclusion bearing cells have been reported in endocervical biopsies (Goldman et al, 1969; Ross, 1966; McCracken et al, 1974).

CMV was isolated by Melnick et al (1978) from two of 10 cell cultures derived from cervical cancer biopsies from patients in an advanced stage of the disease. The two CMV positive biopsies were from two elderly women who had been sexually inactive for many years and therefore suggested persistent latent CMV infection with intermittent periods of activity, rather than recent veneral transmission.

Human papilloma virus

Condyloma acuminatum is relatively common in patients attending venereal disease clinics (Skinner, 1976), and is venereally transmitted (Theokarov, 1969; Oriel, 1971). Evidence has been shown that the virus causing genital condyloma is immunologically distinct from that causing non-genital condyloma. The virus is morphologically identical to SV40 and polyoma virus which are oncogenic in certain animals and morphological transformation of primary human embryonic skin cells by pooled human wart material has been reported (Noyes, 1965). Penile carcinomas have been observed to develop close to or at a site of condyloma acuminata (Sims and Garb, 1951). The cervix is less frequently involved than the vulva (Oriel, 1971). It is a virus which has received little attention in epidemiological and serological studies relating to cervical, penile, vulval or perianal carcinomas (zur Hausen, 1976).

Discussion of studies on the role of other venerally transmitted agents

The spermatozoan theory is a startling one. It dares to link an agent as universal as a sperm with the development of cervical carcinoma. The studies of Coppleson and those of Singer (page 73) on the differences in the transformation zone of the cervical epithelium in sexually active adolescents and virgins were unfortunately carried out on girls in a state institution and in prison respectively. Although the authors do not say so, it is possible that

sexually active adolescents may have been promiscuous and may have had previous venereal disease. Therefore, it is difficult to determine from their experiments whether the abnormal transition zone results from the action of a sexually transmitted infective agent or from the action of spermatozoans. (The abnormal transition zone is believed to harbour potentially malignant cells.) A recent study (Sumithran, 1977) on the rarity of cervical carcinoma among the Malaysian Orang Asli (Aboriginal group) is helpful in clarifying this. Of 18,000 females seen at the Gombak Orang Asli Hospital over a 13 year period, only three patients were diagnosed as carcinoma of the cervix. Interviews with 200 Orang Asli females showed their mean age of marriage to be 14 years, mean age of first pregnancy 16 years, and mean number of pregnancies five. Ninety per cent of the women were married only once, 14% used contraceptives of which none were barrier methods and none of the husbands were circumcised. The tribe followed a strict moral code which is applicable to both males and females and has a low incidence of venereal infection. Since in these women adolescent marriage and childbearing and the non-usage of barrier contraception would have resulted in the exposure of their cervices during metaplastic periods to the action of spermatozoans, this study argues against the spermatozoan theory, and supports the aetiological action of another venereally transmitted agent in the pathogenesis of the cancer. Returning to the Coppleson and Singer studies on sexually active adolescents, it is possible that the abnormal transformation zones in these girls resulted from the action of a sexually transmitted agent other than spermatozoa, i.e.

such an agent was the initiator of the malignant process which is believed by some to develop from groups of cells in the abnormal transformation zone. Up to date research efforts have concentrated mainly on the role of HSV-2 in cervical carcinoma. However the studies quoted above indicate that Chlamydiae, mycoplasma and cytomegalovirus may play a role in cervical anaplasia, that gonococci may act as co-agents, and that the role of human papilloma virus has not been investigated at all.

Chapter 2

THE HERPES SIMPLEX VIRUS

CHAPTER 2

THE HERPES SIMPLEX VIRUS

Classification

Herpes simplex virus belongs to the family of Herpes viruses which are relatively large ether sensitive DNA viruses, and to the subfamily of Alphaherpesvirinae (provisional classification of herpes viruses, 1977).

Morphology of the herpes simplex virus

The virus has an icosahedral capsid with 162 capsomeres on the surface (Wildy et al, 1960). Each capsomere is a hollow prism 12.5 - 9.5 nm in diameter. The core within the capsid is doughnut shaped and consists of proteins around which DNA is coiled (Furlong et al, 1972). The envelope consists of lipid and polyamines (Gibson and Roizman, 1971) and virus specified glycoproteins (Heine et al, 1972). Particles may be found with or without the core or envelopes (Watson et al, 1963). Naked particles have a diameter of 100 nm (Aurelian, 1974a) and the enveloped particles a diameter of 140-160 nm (Nahmias and Roizman, 1973). There is evidence to suggest that naked particles are not infectious (Gibson and Roizman, 1972; Roizman, 1969).

Replicative and metabolic events in a herpes simplex virus infected cell

Following entry of the virus into the cell, the outer protein coat is removed and the DNA protein complex enters the nucleus, where most of the subsequent events occur (Roizman and Furlong, 1974). Viral DNA is transcribed and the membrane bound polyribosomes in the cytoplasm synthesize

structural and functional proteins which are coded for by the mRNA. Most of the proteins migrate back to the nucleus and comprise three sequentially synthesized, co-ordinately regulated groups designated α,β and γ (Honess and Roizman, 1974; Roizman and Morse, 1977). A few proteins remain in the cytoplasm and bind to cellular membranes which become antigenically modified.

α polypeptides

These polypeptides reach peak production two to four hours after infection and three have been recognised as being virus specific - infected cell protein (ICP) 4, 0 and 27. Prior protein synthesis is not required for the formation of α polypeptides.

β polypeptides

These require the presence of αpolypeptides for their synthesis. Viral DNA polymerase, thymidine kinase, and proteins responsible for the inhibition of cellular macromolecular metabolism and of polypeptide synthesis, comprise the βpolypeptides.

γ polypeptides

These require the presence of βpolypeptides for their synthesis and comprise the structural polypeptides of the virus. The rate of production of γpolypeptides increases up till 15-18 hours post infection.

Thirty of these polypeptides have now been mapped on

the herpes genome by Roizman's group (Unpublished - presented at herpes virus workshop, Cambridge, 1978).

The transition from α to β polypeptide synthesis is in most cases independent of viral DNA replication (Wolf and Roizman, 1977). The γ messenger RNA's have a short functional half life compared to the α and β mRNA's which have a considerably longer half life. For this reason some polypeptides are not detectable in cells infected at low multiplicity in which the synthesis of viral progeny DNA is inhibited. On infecting such cells at high multiplicity, however, these polypeptides are seen in larger quantity. The shorter half life of the polypeptide mRNA is thought to provide an effective method or regulating viral assembly in infected cells. Thus in the early stages of infection the rate of synthesis of structural proteins would be low and would be accelerated when the pool of DNA increases by DNA replication. This also explains why on inhibiting DNA synthesis cells infected with some human herpes viruses do not possess structural viral components.

New virus capsids are formed in the nucleus. Envelopment occurs at the inner lamella of the nuclear membrane and sometimes also at the cytoplasmic membrane (Strandberg and Aurelian, 1969). Virus release has been described as a process of inverted pinocytosis (Morgan et al, 1959).

DNA of the herpes simplex virus

The DNA of the virus is a linear double stranded molecule 100 x 10^6 daltons in molecular weight (Keiff

et al, 1971) which is larger than the DNA molecules of some of the other non-herpes oncogenic DNA viruses (Table 2.1). Each molecule consists of two unique components UL and US, each of them bound by an inverted repetitive region (Roizman and Morse, 1977).

The UL component and the repetitive regions bounding it comprise 82% of the molecule and the US region and its repetitive regions comprise 18% of the molecule.

Fifty per cent of the viral DNA is expressed during a productive infection (Roizman and Frenkel, 1973). From the size of the DNA molecule it was estimated that it could code for approximately 65 proteins (Nahmias and Roizman, 1973). Forty-seven have now been identified of which 27 are structural (Aurelian, 1974a). The DNA's of HSV-1 and HSV-2 show 47% homology by nucleic acid hybridization (Keiff et al, 1972). Therefore most antigens coded for by the two viruses are type common.

The physical state of virus DNA in cells transformed by oncogenic viruses

In cells infected as well as those transformed by RNA tumor viruses, papovaviruses, and adenoviruses the viral DNA is carried in a linearly integrated form (Martin and Khoury, 1976; Pitot, 1978). In some cells both integrated DNA as well as non-integrated circular viral DNA has been reported (Prasad et al, 1976; Ringold et al, 1977).

Table 2.1 Properties of the viral genome in oncogenic viruses (adapted from Pitot, 1978)

VIRUSES	MOLECULAR WEIGHT OF GENOME	STATE OF DNA IN VIRUS	CODING POTENTIAL (CALCULATED NO. OF POLYPEPTIDES)	PHYSICAL STATE OF VIRUS DNA IN INFECTED AND TRANSFORMED CELLS
Papilloma - myxoma viruses	$\underline{c}$ 160 x 10^6	circular	-	-
Herpes simplex virus	100 x 10^6	linear	65	Not known
Cytomegalovirus 2 molecular weight classes*	100 x 10^6 150 x 10^6	linear	-	Not known
Epstein-Barr virus	106 x 10^6	linear	-	Integrated and episomal forms in transformed and tumor cells
Adenoviruses	20-25 x 10^6	linear	23	Integrated to cellular DNA in infected cells as well as transformed cells
Polyomaviruses (SV-40, polyoma, JC and BK)	3 x 10^6	linear	7 (?)	Integrated to cellular DNA in infected cells as well as transformed cells
Rouse sarcoma virus (C-type particles)	3 x 10^6	linear	7 (?)	Integrated to cellular DNA in infected cells as well as transformed cells
Mammary tumor virus (B-type particles)	3 x 10^6	linear	7 (?)	Integrated to cellular DNA in infected cells as well as transformed cells

* Kilpatrick and Huang (1977)

Experimental techniques employed in work of this nature depends on the size difference between free virus DNA and cellular DNA. Such techniques are not easily applicable to the herpes viruses with DNA of molecular weight 100 x 10^6. Therefore, although virus DNA has been demonstrated in the cells transformed by many herpes viruses, by means of nuclear hybridization techniques, the physcial state of the DNA has been partly established only in the case of the EB virus, herpes virus siamiri and the herpes virus papio. EBV DNA was found in Raji cells (cells established from a Burkitt's lymphoma (BL) patient) as well as BL tumor cells and nasopharyngeal carcinoma (NPC) tumor cells, mainly in the form of circular episomal DNA (Adams and Lindahl, 1975; Kaschka Dierich et al, 1976). Integrated sequences were also found in Raji cells (Adams et al, 1973) and BL tumor cells and NPC tumor cells (Kaschka Dierich et al, 1976). However, although in these cells the episomal DNA had a molecular weight similar to virus DNA, it is not known how much of the viral genome the integrated viral DNA represents. B cells from a case of infectious mononucleosis had episomal DNA which was smaller than the episomal DNA from BL tumor cells (Adam et al, 1977). The episomal DNA in these tumors is similar to episomes in bacteria. It is possible that all herpes viruses may be carried as episomes during latency. The difference in size of the episomal DNA in infectious mononucleosis and BL and NPC tumors suggests that the host cell determines partly the physical state of the viral DNA in different tumor cells associated with the same virus.

Similarities and differences between HSV-1 and HSV-2

Table 2.2 is a summary of some studies which have demonstrated biological, antigenic, genetic and protein differences between HSV-1 and HSV-2.

The latent herpes simplex virus

The clinical features of recurrent herpes simplex infection point to it being caused by endogenous rather than exogenous virus, because a) the site of recurrence is usually the same or a closely neighbouring site, and b) it is often associated with a particular event, e.g. fever, injury, exposure to sunlight in the case of oro-facial lesions, or menstruation, coitus, masturbation in the case of genital herpes. The patient is often able to predict when a recurrence is likely. Reactivation of herpes simplex infection is also common in immunosuppressed patients, e.g. due to underlying disease such as Hodgkin's disease or deliberate immunosuppression during organ transplantation.

Roizman (1965) has suggested two possible states of latency:

1. The dynamic state.
2. The static state.

The dynamic state hypothesis postulates that low grade multiplication of the virus continues between recurrences. The static state hypothesis postulates that the virus remains in a non-infectious form at or close to the site of

Table 2.2 Similarities and differences between HSV-1 and HSV-2

REFERENCE	PARAMETER OF COMPARISON	HSV-1	HSV-2
Dowdle et al (1967)	Site of infection	Primarily non-genital	Primarily genital
Dowdle et al (1967)	Mode of transmission	Primarily non-venereal	By sexual contact or genital contact, eg newborn
Parker and Banatvala (1967)	Pock size on CAM - 7 days incubation	Small pocks	Large pocks 3-5 mm in diameter
Dawson (1933)	Histological findings on CAM	Primarily ectodermal involvement	Involves all membrane layers. Syncitia more common.
Nahmias and Dowdle (1968)	Cytopathic effect on HeLa cells	Tight adhesions of rounded cells	Loose aggregates of rounded cells, syncitia common
Ratcliffe (1971)	Effect of temperature on growth in tissue culture	Replication at 39.8°C and 40.3°C	Marked inhibition of growth at temperatures above 39°C
Mogenson (1976)	Necrotic hepatitis test in mice. I.P. injection of HSV.	Few tiny lesions in the liver	Numerous necrotic lesions in the liver
Marks-Hellman and Ho (1976)	Heparin sensitivity estimated by plaque reduction	Sensitive to heparin	Insensitive to heparin
Thouless and Skinner (1971)	Thymidine kinase induced in infected cells	Stable at 4°C	Unstable at 4°C
Thouless (1972)	Thymidine kinase	Enzymes induced by HSV-1 and HSV-2 had more type-specific determinants than type common.	
Halliburton (1972)	Density of DNA	HSV-2 DNA had a greater density than HSV-1 DNA indicating a 2.2% difference in G.C. content, i.e. a difference of 3850 or more base pairs.	
	Viral proteins	Difference in the size of 3 viral proteins.	

Table 2.2 (cont'd)

REFERENCE	PARAMETER OF COMPARISON	HSV-1	HSV-2
Kieff et al (1972)	Comparison of DNA by molecular hybridization	50% of sequences were common to both.	
Honess et al (1974)	Antigens on the surface of naked particles and enveloped particles by immune agglutination.	Type specific and type common antigens detected on both the surface of the virus capsid as well as the envelope.	
Schwartz and Roizman (1969)	Naked and enveloped particles in infected cells.	More naked particles observed in the cytoplasm of HSV-2 infected cells than HSV-1 infected cells.	
Cassai et al (1975)	Proportion of naked particles in purified virion preparation.	10%	30%
Cassai et al (1975)	Polypeptides of purified HSV-1 and HSV-2 by SDS acrylamide gel electrophoresis	1) Both viruses contained approximately equal numbers of polypeptides. 2) Major capsid protein of HSV-2 migrated slower than the major capsid protein of HSV-1 3) At least 3 glycoproteins of HSV-2 differed from HSV-1 glycoproteins. 4) 2 prominent HSV-1 polypeptides did not have electrophoretically similar counterparts among the polypeptides of HSV-2 isolates.	

recurrence. In support of the dynamic state hypothesis are the reports of isolation of the virus from the male genitourinary tract (Centifanto, 1972) and the uterine cervix (Rattray et al, 1978) between recurrent episodes. However, skin biopsies taken between attacks from the sites at which recurrences occur failed to yield infectious virus (Roizman, 1965). In support of the static state hypothesis is the isolation of HSV-1 from the trigeminal and sacral ganglia of human cadavers (Bastian et al, 1972) and sacro-sciatic spinal ganglia of mice infected via their foot pads (Stevens and Cook, 1973). The virus present in these nerve ganglia could not be cultured by the usual straight-forward isolation methods, indicating that it was not present in the infectious form.

It is necessary for the survival of the virus lying latent in its host cell that it should not inhibit host cell macromolecular synthesis, i.e. the translation of its DNA needs to be arrested before its α polypeptides are formed. The questions which arise are:

1. Is this switch off of viral DNA translation virus-coded or host-coded?
2. Is there any viral expression in the latent state, e.g. are the α polypeptides formed?
3. If the switch off is host cell coded, are recurrences due to these host cells being subject to stress, thereby altering or diminishing their normal function? Similar expression of the EBV in lymphoblastoma cells which were stressed by the use of 5-iododeoxyuridine has been described (Hampar et al, 1972).

These questions about virus latency have not yet been answered. In addition it is not known whether there are sites besides the nerve ganglia in which virus latency occurs, or whether within the nerve ganglion the virus is latent in the neurones or the supporting cells. It has also not been established whether the viral DNA lies in a free episomal state or in an integrated form. Pagano (1975) has suggested that the genome which is reactivated in recurrent infection may be in an episomal state and that which is responsible for malignant change (if any) in an integrated state.

Chapter 3

THE UTERINE CERVIX & ITS EPITHELIUM

CHAPTER 3

THE UTERINE CERVIX AND ITS EPITHELIUM

The cervix is the lower part of the uterus, the upper limit of which is the internal os (Figure 3.1). It opens into the vagina by the external os. Between the internal os and the external os lies the endocervical canal. The shape of the cervix is basically cylindrical and the vaginal portion (ectocervix) consists of an anterior lip and a longer posterior lip. The vaginal mucosa is reflected onto the ectocervix anteriorly, laterally and posteriorly forming the vaginal fornices. Anteriorly, the supravaginal portion is separated from the bladder by a layer of connective tissue called the parametrium. The parametrium extends laterally into the space between the two layers of the broad ligament. Posteriorly, the supravaginal portion of the cervix is covered with peritoneum which continues down over the posterior vaginal wall to be reflected onto the rectum, thereby forming the pouch of Douglas.

In late fetal life the original columnar epithelium derived from the Mullerian duct covers the uterine cavity and extends downward through the cervical canal where it meets the original squamous epithelium lining the vagina which is derived from the urogenital sinus. The boundary between these two types of epithelium occurs at an easily recognisable point called the squamocolumnar junction. This could be at any point across the ectocervix or endocervical canal, or in a small proportion of women (4%) in

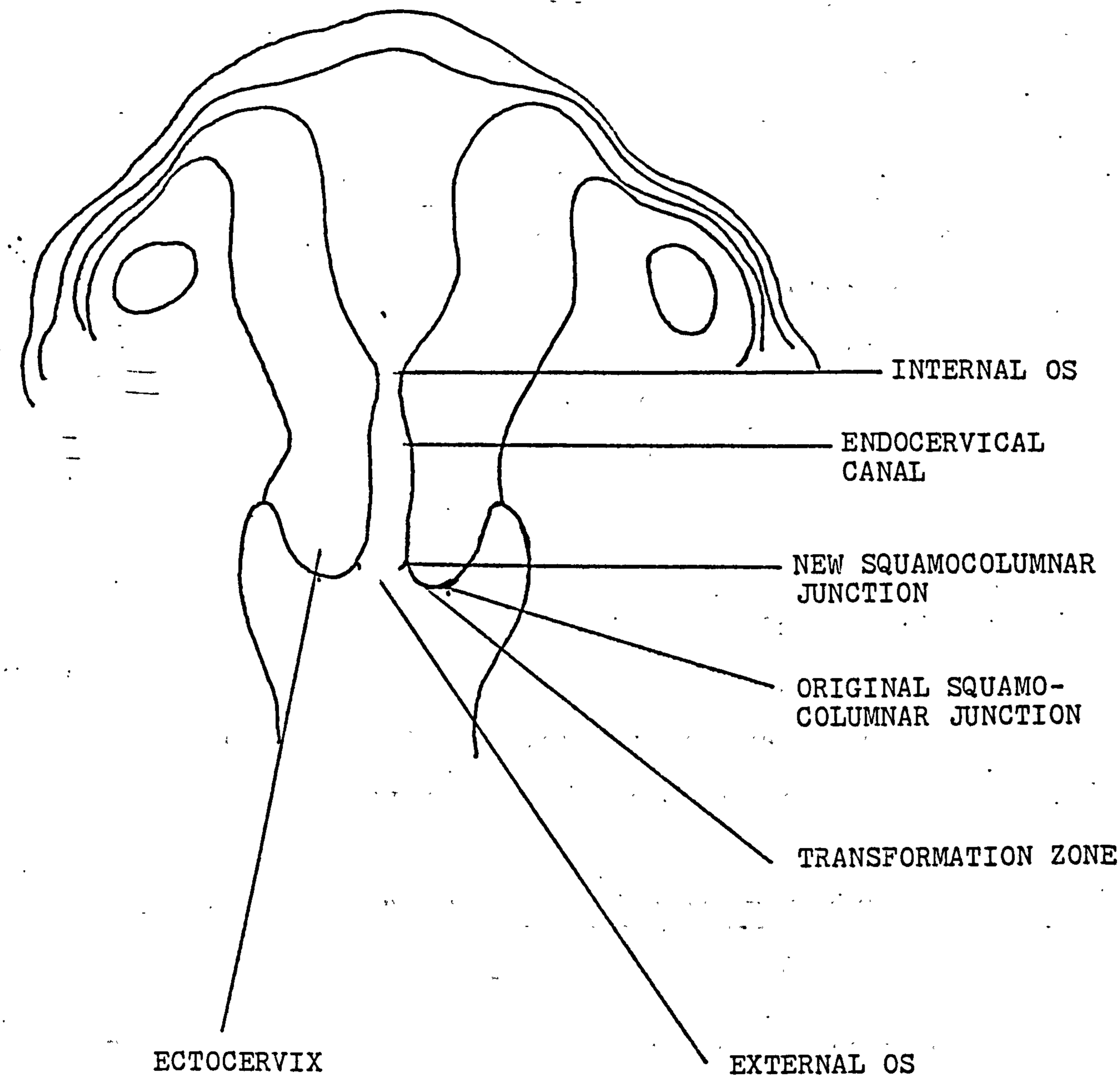


Figure 3.1 Cross-sectional diagram of the uterus and cervix.

the vaginal fornix (Singer and Jordan, 1976). During late fetal life, adolescence and following first pregnancy, the more distal portion of the original columnar epithelium is partly or wholly replaced with squamous epithelium by a physiological process called squamous metaplasia. This process occupies a short period of time measureable in days or weeks and its natural history has been studied by the use of the colposcope. The new squamous epithelium laid down is called metaplastic epithelium. It lies between the old squamocolumnar junction and the new squamocolumnar junction and is termed by colposcopists the transformation zone. In some women a colposcopically normal transformation zone is seen which is termed the physiological transformation zone. In others there is a colposcopically abnormal transformation zone which is termed the atypical transformation zone.

THE EFFECT OF COITUS DURING EARLY ADOLESCENCE ON THE COLPOSCOPIC APPEARANCE OF THE TRANSFORMATION ZONE

Epidemiological studies have repeatedly demonstrated the importance of coitus in relation to the aetiology of cervical carcinoma (Rotkin, 1967 (a), and (b) and 1973). Copplesson *et al* (1975) and Singer (1975) studied the appearance and size of the transformation zone in sexually active adolescents and virginal adolescents (Table 3.1). There was a higher incidence of both an atypical transformation zone and retraction of the new squamocolumnar junction into the endocervix in the sexually active, in comparison with the virginal girls. There was also a

Table 3.1 The effect of coitus during early adolescence on the colposcopic appearance of the transformation zone

Reference	Study group	Mean age at 1st coitus	Proportion having an atypical transformation zone	Proportion having a physiological transformation zone	Retraction of squamocolumnar junction into endocervical canal	Total area of the transformation zone
Coppleson et al (1975)	170 sexually promiscuous nulliparous girls under 17 years, in a state institution	13.4 (± 2.7) 14% prior to menarche	75 (44%)	80 (48%)	15 (8%)	117 mm^2
	40 virginal Australian girls of mean age 19 years		5 (12.5%)	29 (72.5%)	2 (5%)	
Singer (1975)	168 nulliparous women under 21 yrs in a London prison	14.9 (± 3.2) 10% prior to menarche	70 (42%)	60 (35%)	38 (23%)	not measured but an impression of a smaller transformation zone
	40 virginal girls					170 mm^2

significant difference (P<.01) between the size of the transformation zone observed in sexually active adolescents in the Coppleson study in comparison with that of virginal girls in the Singer study (Singer, 1976). These findings are of possible relevance to the aetiology of cervical carcinoma. It has been suggested by Coppleson and Reid (1967 and 1975) that in the process of the development of an area of atypical metaplasia a mutation may be induced in the epithelium and subepithelial area which endows it with a neoplastic potential.

MICROSCOPIC APPEARANCE OF THE CERVICAL EPITHELIUM

Microscopically the squamous epithelium consists of five layers of cells which are separated from the fibrous stroma by a basement membrane.

1. Basal cell layer This is the deepest layer which lies adjacent to the basement membrane, consisting of a single row of small cylindrical cells with relatively large nuclei.

2. Parabasal cell layer (Prickle cell layer) This consists of several layers of polyhedral cells with fairly large nuclei.

Mitotic figues are occasionally found in these two layers.

3. Intermediate cell layer (Navicular cells) A layer of cells which begins to show flattening and have a cytoplasm rich in glycogen with frequent vacuolation.

4. Intraepithelial layer (Condensation zone) A layer of closely packed polyhedral cells with keratohyalin granules.

5. Stratum corneum Elongated flattened cells with small nuclei and a large amount of cytoplasm.

CLASSIFICATION OF CERVICAL SMEARS

As suggested by Papanicolaou, cervical smears are classified into five categories (Wachtel, 1969).

Grade 1 - All normal smear patterns.

Grade 2 - (2N) Abnormal but benign types of cells as well as inflammatory smear patterns.

Grade 3 - (3A or 3B) Abnormal cells falling short of the description of a malignant cell but sufficiently different from the normal to create apprehension. 3A smears are mild dysplasias and 3B a moderate - severe dysplasia.

Grade 4 - (Positive 4) (Carcinoma in situ or invasive carcinoma) These are smears showing a few malignant cells. At times they include cases of severe dysplasia. The differentiation between a severe dysplasia carcinoma in situ and an invasive carcinoma is made on histological examination of biopsy material.

Grade 5 - Large numbers of malignant cells are seen in these smears.

ABNORMAL FEATURES MOST FREQUENTLY SEEN IN MALIGNANT CELLS (WACHTEL, 1969)

1. Nuclear changes

a) There is gross enlargement of the nucleus without corresponding enlargement of the cell body. The nuclear/cytoplasmic ratio is disturbed, the nucleus filling at least one-third of the cell volume.

b) The nuclei lose their smooth rounded shape and assume an irregular outline. They also appear in a variety of sizes.

c) Whereas the chromatin of normal cells is evenly distributed and appears smooth or finely granular, malignant cells have irregular chromatin distribution giving a rough irregular appearance.

d) Additional features such as hyperchromasia, multinucleation and enlargement and/or reduplication of nucleoli are often but not invariably observed.

2. Cytoplasmic changes in malignant cells

The cytoplasmic changes in malignant cells are less distinctive and may also be found in severe inflammatory disease, metaplasia or under the influence of radiation.

a) Alteration in staining properties.

b) Cellular enlargement and bizarre cell shapes.

c) Cellular inclusions.

d) Degenerative changes.

STAGING OF CARCINOMA OF THE CERVIX

The disease is staged for purposes of international classification and therapy according to the degree of spread. The classification currently in operation is the one laid down by the International Federation of Gynaecology and Obstetrics (F.I.G.O. News, 1965).

Stage 1a - Subclinical lesion which is minimally but clearly invasive, i.e. extends deeper to the basement membrane.

Stage 1b - Clinical lesion - confined to the cervix or extending only to the corpus.

Stage 2a - Extends to the vagina but not the lower third.

Stage 2b - There is parametrial involvement which does not extend to the pelvic wall. Vaginal involvement but not to the lower third.

Stage 3a - Involvement of the lower third of the vagina.

Stage 3b - Involvement of pelvic wall.

Stage 4 - Involvement of the bladder or rectal mucosa or presence of distant metastases.

Chapter 4

INTRODUCTION

CHAPTER 4

INTRODUCTION

INCIDENCE OF CERVICAL CARCINOMA IN BRITAIN, SRI LANKA, MALAWI AND SUDAN

Cervical carcinoma is the commonest genital tumor in the United Kingdom (Chamberlain and Dewhurst, 1977. Table 4.1), Sri Lanka (Fernando, 1974. Table 4.2), Malawi (M.S.R. Hutt, Personal communication. Table 4.3) and one of the commonest tumors in females in Sudan (M.S.R. Hutt, Personal communication).

It is well established that various population groups either show a low risk or a high risk of developing cervical carcinoma. Table 4.4 demonstrates the variation in incidence of the disease among women in different countries. Attempts have been made to search for a pattern of epidemiological and aetiological variables which might account for the enormous difference.

SOME SOCIAL ASPECTS RELATED TO CERVICAL CARCINOMA

Table 4.5 contrasts the incidence of carcinoma of the cervix among different groups of women sharing the same environment. The study done by Stewart *et al*. (1966) showed a striking difference in the incidence rates in New York City between Puerto Rican women (105.7/100,000 population), Negro women (49.6/100,000), non-Jewish white women (15/100,000) and Jewish women (4.1/100,000).

Table 4.1 (From Chamberlain and Dewhurst, 1977)

Gynaecological diagnoses of patients admitted to National Health Service hospitals in England and Wales in one year recently

Malignant tumors	Cervix	14,650
	Body of uterus	4,520
	Not stated (uterus)	2,470
	Ovary and Tube	6.670
	Other genital	2,240
		30,550
Benign tumors	Fibroids	26,640
	Ovary	12,430
	Other genital	4,270
		43,340

Table 4.2 (From Fernando, 1974)

An analysis of 17,277 cases with female genital cancer admitted from 1968 - 1972 to the Cancer Institute, Maharagama, Sri Lanka

	Number of cases	% of all admissions
Carcinoma of cervix **	2316	13.4%
Carcinoma of ovary *	189	1.9%
Carcinoma of vagina	86	0.49%
Carcinoma of vulva	52	0.306%
Carcinoma of body of uterus *	46	0.26%
Carcinoma of Fallopian tube	0	0%

* Seen less frequently in the series as they are treated in other hospitals as well

** Early stages are treated by surgery alone in other hospitals

Table 4.3 (M.S.R. Hutt, Personal communication)

Incidence rates (per 100,000 per year) of carcinoma of the cervix, bladder, skin and breast in females in Malawi of the age group 35-64

	MALAWI
Carcinoma of the cervix	79.6
Carcinoma of the bladder	30.8
Carcinoma of the skin	26
Carcinoma of the breast	23

Table 4.4 (Adapted from Coppleson, 1969)

Incidence of carcinoma of the cervix in some countries - world standard rate (WSR) for all ages

Country	World Standard Rate
Columbia, Cali	100.6
S..Africa (Bantu)	52.0
Hamburg	36.2
Denmark	28.3
Slovenia	26.2
Singapore (Chinese)	21.4
Sweden	17.2
England (4 regions)	16.2
Norway	15.3
Iceland	15.3
New York State	14.6
Connecticut	13.6

Table 4.5

Groups of women found to be at relatively low and high risk of developing cervical carcinoma

Groups at low risk	Groups at high risk
Jewish women in New York City (Stewart et al, 1966)	Puerto Rican women and Negro women in New York City (Stewart et al, 1966)
Irish and Italian immigrant women in the USA (Haenszel, 1961)	
Protestant and Catholic women who regularly attend religious services in Washington County, USA (Naguib et al, 1966)	Protestant and Catholic women who rarely or never attend religious services in Washington County, USA (Naguib et al, 1966)
Seventh Day Adventist women (Wynder et al, 1959)	
Malaysian Orang Asli (Sumithran, 1977)	Chinese and Indian women in Malaysia (Marsdon, 1958)
	Prostitutes (Rojel, 1953 and Pereyra, 1961)

In a study conducted in the Washington County in the USA, Naguib et al (1966) found a higher incidence of abnormal cervical smears among Protestant and Catholic women who were not practicing Christians in contrast to those who were. Similarly, Haenszel (1961) found a low incidence among Irish and Italian immigrant women in the USA who were Roman Catholics, and Wynder et al (1959) a low incidence among Seventh Day Adventist women. Sumithran (1977) reported a very low incidence among the Malaysian Orang Asli in comparison with the Chinese and Indian women in Malaysia in whom Marsdon (1958) reported cervical carcinoma to be the commonest cancer. These differences may be due to the effect that such influences as cultural background, socioeconomic status and religious commitment have on individual attitudes towards sexual conduct. Thus Jewish doctrine, Roman Catholicism, The Seventh Day Adventists, and many Protestant deominations preach a conservative attitude towards sexual conduct. In addition among Jews, another relevent cultural factor is the practice of one of the Mosaic laws, the law of Niddah relating to abstinence from coitus during and immediately following mentruation and childbirth (see page 48). A feature among the Malaysian Orang Asli which is stressed by Sumithran is their custom of early marriage and their strong disapproval of adultery. This combination results in strictly monogamous relationships. Reports of the high incidence of carcinoma of the cervix among prostitutes (Rojel, 1953; Pereyra, 1961. Table 4.5) contrasts with the above findings.

Of the four countries from which samples were collected for the studies reported in this thesis, religion has considerable influence in the behavioural characteristics of the people in Sri Lanka and Sudan. Permanency of marriage is favoured and pre-marital and extra-marital sex frowned upon. This same degree of influence of religious ideals on sexual behaviour is not seen in Malawian and British society.

Ethnically, Sudan and Malawi provide a relatively homogeneous population, in contrast to Britain (Caucasian, Negroid and Asian) and Sri Lanka (Sinhalese, Tamil, Muslim and other).

The social class structure of Malawi, Sudan, and Sri Lanka has not been classified as exhaustively as for Britain (Office of the population censuses and surveys, 1970). However there are accepted low, middle and upper classes in these countries and the proportion of people falling into the middle and upper classes are approximately 1% in Malawi, 10% in Sudan, and 15% in Sri Lanka.

RELEVANCE OF SURFACE ANTIGENS IN CERVICAL CARCINOMA

Much of the normal protective work of the immune system is directed against cell surface antigens, and depends on cell surface receptors. This has found important application in many fields of medicine, e.g. blood grouping, tissue matching, control of haemolytic disease of the newborn and immune tolerance. The growing importance of oestrogen receptors in deciding treatment strategy for breast cancer (McGuire, 1978), and glucocorticoid receptors in human leukaemia (Lippman et al, 1978) are two of the many possible applications in the future.

New antigens have been demonstrated on the surface of cells infected by non-oncogenic, as well as oncogenic viruses (Table 4.6). These antigens are virus specific (Hyashi et al, 1972). Table 4.7 shows some of the techniques used to demonstrate virus specific antigens on cells infected by some of the herpes group of viruses. They were first demonstrated on cells infected by Herpes simplex viruses by O'Dea and Dineen (1957) using an indirect fluorescent test on live cells. Their time of appearance after infection, as demonstrated by different techniques, ranges from two hours (Tevethea et al, 1977) to six hours (Lowry et al, 1971) (Table 4.8). Also explained in the same table is the use of Actinomycin D, IUDR, cytosine arabinoside and cycloheximide in establishing that the surface antigens are coded for at least partly, by infecting DNA and do not require the prior production of progeny DNA. Herpes simplex induced membrane antigens

have not been sub-typed into early and late components as have the EB virus coded membrane antigens (Epstein and Achong, 1977). It has been established however that they are composed of antigens common to HSV-1 and HSV-2 as well as antigens specific for each (Nahmias et al, 1971a, and Geder and Skinner, 1971).

Table 4.6 Demonstration in vitro of virus specific antigens on the surface of infected cells (modified from Table by Burns and Allison, 1977)

VIRUS GROUP	VIRUS	ASSAY
Herpes viruses	Separate table with references	
Togaviruses	Chikungunya	IF
	VEE	IF
	EEE	CT
	Sindbis	IF, LC
	Semliki Forest	IF, CT, LC
	Dengue	CT, IEM
	JEE	CT
Orthomyxoviruses	Influenza	IEM, CT, IF
Paramyxoviruses	Mumps	IEM, LC, IF
	Sendai	CT
	SV 5	CT
Probable Paramyxoviruses	Measles	IF, LC
	Rinderpest	IF, LC
	Canine distemper	IF, LC
Oncornaviruses	Murine leukaemia	CT, IEM, LC
	Murine mammary tumor	IA
	Feline leukaemia	IEM, RI
	Avian Tumor	IEM, LC
Rhabdoviruses	Vesicular stomatitis	IEM
	Rabies	CT
Arenaviruses	Lymphotic chorio-meningitis	LC, IF, IEM
Adenoviruses	Type 12	IF, LC
Papovaviruses	SV 40	CT, RI
	Polyoma	IF
Pox viruses	Vaccinia, cow pox, monkey pox	IF, IA, CT, RI
	shope fibroma	IF, CT

IF Immunofluorescence CT Complement dependent cytotoxicity
LC Lymphocyte mediated cytotoxicity RI Radioimmunoassay
IA Immune adherence IEM Immune electron microscopy

Table 4.7 Demonstration in vitro of virus specific antigens on the surface of cells infected by members of the herpes group of viruses (modified from a table by Burns and Allison, 1977)

VIRUS	TECHNIQUE	REFERENCE
Herpes simplex	IF	O'Dea and Dineen (1957)
		Nahmias et al (1971a)
		Espmark (1965)
	MHA	Ito and Barron (1972)
		Espmark (1965)
	CT	Roane and Roizman (1964)
	IEM	Nii et al (1968)
	RIA	Hyashi et al (1972)
Varicella-zoster	IF	Gershon et al (1974)
CMV	IF	deThe and Langethuysen (1972)
EB	IF	Klein et al (1968)
Marek's disease	IF	Chen and Purchase (1970)

IF Immunofluorescence MHA Mixed haemagglutination

CT Complement dependent cytotoxicity

IEM Immune electron microscopy RIA Radioimmunoassay

Table 4.8 Time of appearance of membrane antigens (MA) of HSV infected cells, as detected by different techniques. Effect of inhibitors of DNA synthesis and of protein synthesis on their formation.

REFERENCE	TECHNIQUE	TIME OF APPEARANCE OF MA AND VIRUS AFTER INFECTION	ACTION OF INHIBITORS OF DNA SYNTHESIS AND PROTEIN SYNTHESIS	CONCLUSION
Watkins (1965)	Haemadsorption	MA - 5 hours virus - 12 hours	IUDR* diminished number of haemadsorbing cells if added up to 8 hours but did not totally prevent formation of MA. Actinomycin** prevented formation of MA if added from 1-3 hours after infection. Progressively diminished effect on production of MA if added from 4 hours onward.	Surface changes detected by haemadsorption are coded for by infecting DNA
Nahmias et al (1971a)	Indirect immuno-fluorescence	Not given	IUDR, Cytosine Arabinoside membrane antigen formed	Confirmation of findings by Watkins
Lowry et al (1971)	Immuon-fluorescence	6 hours		
Present study	Indirect immuno-fluorescence	5 hours		

Table 4.8 (continued)

REFERENCE	TECHNIQUE	TIME OF APPEARANCE OF MA AND VIRUS AFTER INFECTION	ACTION OF INHIBITORS OF DNA SYNTHESIS AND PROTEIN SYNTHESIS	CONCLUSION
Ito and Barron (1972)	Mixed haemadsorption	MA in a few cells at 4 hours. 100% of cells at 8 hours. Virus - 8 hours.	Cytosine arabinoside*** no effect on formation of MA and no viral progeny formed. Cycloheximide**** Totally inhibited formation of MA. No viral progeny formed.	MA formation does not require synthesis of new viral DNA.
Tevethia et al (1972)	Agglutination with concanavalin A.	2 hours - 2 + agglutination 4 hours - 4 + agglutination	Cycloheximide Added at time of infection completely prevented formation of MA. Added 4 hours after infection partially diminished formation.	Surface changes occur as early as 2 hours after infection although not detected by other techniques.

* IUDR - interferes with thymidine phophorylation and thereby with DNA synthesis but has no effect on protein synthesis.

** Actinomysin D - prevents DNA dependent RNA synthesis

*** Cytosine arabinoside - blocks viral synthesis

**** Cycloheximide - inhibits protein synthesis

MA Membrane antigens

NATURE OF MEMBRANE ANTIGENS ON HSV INFECTED CELLS

New antigens induced by viral infection are either virus specified (virion or non-virion) or host specified. They remain unaltered or are modified by glycoselation by host enzymes and inserted into cell membranes (Burns and Allison, 1977).

VIRUS CODED ANTIGENS ON THE SURFACE OF HSV INFECTED CELLS

a) Envelope antigens

Lesso et al (1970) demonstrated that rabbits inoculated with a membrane preparation from HSV, developed neutralizing antibody in high titre. Smith et al (1972b) found a good correlation between antibody to membrane antigen of HSV-1 and HSV-2 infected cells and neutralizing antibody. These findings support the view that a proportion of the antigens on the surface of infected cells are similar to those of the virus envelope.

b) Non-virion antigens

A virion coded soluble non-virion antigen NVA-TAA (non-virion antigen - tumor associated antigen) has been described, associated with the membrane fraction of HSV-1 and HSV-2 cells, which reacts with membrane antigens on carcinoma of the cervix, vagina, vulva and lip (Hollinshead and Tarro, 1973, and Hollinshead et al, 1972). These studies and their relevance to the present study are described in greater detail on page 282.

c) Fc receptors

Receptors for the Fc portion of non-herpes specific IgG have been demonstrated on cells infected or transformed by HSV (Westmoreland and Watkins, 1974, and Westmoreland et al, 1974). These receptors are seen in increased numbers, 8-10 hours post infection, at a time when host cell macromolecular synthesis has been largely turned off. For this reason, they are believed to be viral coded proteins.

HOST CODED MEMBRANE ANTIGENS IN RELATION TO VIRUS INFECTIONS

a) Fetal antigens

Some new membrane antigens are believed to result from derepression of host genes or by the unmasking of antigenic substructures already present, e.g. the demonstration of Forssman antigen on SV-40 and polyoma virus transformed BHK cells (Fogel and Sachs, 1962, Robertson and Black, 1969). The fetal antigen that has received prominence in relation to cancer of the cervix in carcino-embryonic antigen (CEA) (see page 102). The exact location of CEA on the malignant cell has not been described.

b) Histocompatibility antigens

The possibility that new membrane antigens are formed either by viral antigens forming a composite with the H-2 material or by modifying the H-2 antigen is suggested by the behaviour of cytotoxic T-cells of donor mice immunized with LCM virus (Zinkernagel and Doherty, 1974 & 1975).

The T-cell was able to perform lytic activity only if there was some degree of histocompatibility between it and the target cell. Koenig et al (1976) have shown a statistically positive relationship between patients with cervical cancer and the HLA-B-12 type. Could a modification of this antigen by the virus help the cell to escape immune surveillance?

c) Lectin receptors

Infection of cells with HSV brings about in two hours changes in the cell membrane which make the cell more susceptible to agglutination with concanavalin A (Tevethia et al, 1972) (Table 4.8). Concanavalin A is a lectin which is able to agglutinate cells transformed by both RNA and DNA tumor viruses and also normal untransformed cells which have been infected by a variety of viruses (Burns and Allison, 1977).

MEMBRANE ANTIGENS ON HSV TRANSFORMED CELLS

Antiserum prepared against VP 123 and VP 119, the major glycoprotein region of HSV-1 and HSV-2 infected cells respectively, was found to react type specifically with HSV-1 and HSV-2 infected and transformed cells in an indirect immunofluorescence test on live cells (R.J. Courtney, Personal communication). HSV neutralizing antibodies to HSV-1 and HSV-2 were present in sera of newborn hamsters in whom tumors were induced by HSV-1 and HSV-2 transformed cells (Duff and Rapp, 1971; Rapp and Duff, 1973). Since infectious virus could not be isolated from either trans-

formed cells or the induced tumor, it was concluded that antibodies were directed against a membrane antigen on the tumor.

Membrane antigens were demonstrated on cells transformed by mutagenised HSV by a lymphocyte cytotoxicity test (Lausch et al, 1975).

MEMBRANE ANTIGENS ON VIRUS-INDUCED (DNA AND RNA) ANIMAL TUMORS AND IN HUMAN SQUAMOUS CARCINOMAS

Tumor specific transplantation antigen (TSTA) and other membrane antigens have been detected in DNA and RNA induced tumors, e.g. 1) TSTA in SV-40 induced tumors - found only in cells carrying the virus genome, 2) S-antigen also in SV-40 induced tumors found also in cells with no detectable levels of SV-40 m-RNA and SV-40 DNA, 3) TSTA in leukaemic cells caused by mouse leukaemic virus. In this instance the leukaemic cells perpetually synthesize virus particles (Maruyama and Dmochowski, 1973; Tevethia, 1975). NVA-TAA referred to earlier, is an antigen found on the surface of squamous carcinoma cells (cervix, vagina, vulva and lips) which reacts with a herpes virus induced non-virion antigen (Hollinshead et al, 1976). Antibodies to NVA-TAA have been detected in sera from a high proportion of patients with squamous carcinoma (Hollinshead et al, 1973). It also produced a delayed hypersensitivity reaction specific for patients with squamous carcinoma (Hollinshead et al, 1976).

There is growing evidence to indicate the importance

of the humoral antibody response in tumor immunology. Antibodies can either enhance or inhibit tumor growth (Bansal and Sjogren, 1972; Moller, 1964; Ting and Herberman, 1974). With DNA tumor viruses, virus induced, non-virion antigens have been separated from cells undergoing lytic infection by the virus, as well as from tumors (Hollinghead et al, 1968). Hence it is possible that an assay of antibodies to the surface antigens of such a virus infected cell may be also an assay of antibodies to antigens present on an aetiologically related tumor.

At the time this study was begun, three studies had been reported which examined the importance of antibodies to membrane antigens of HSV-2 infected cells in patients with pre-malignant and malignant cervical lesions. Smith et al (1972b) employed indirect immunofluorescence, Thiry et al (1974) a complement dependent cytotoxic antibody test, and Christenson and Espmark (1976) a mixed haemagglutination test. These studies and a subsequent study by Christenson (1977) also employing a complement dependent cytotoxic assay had interesting diagnostic and prognostic implications and have been described on page 322.

Membrane antibodies detected by different techniques may be a test of different populations of these antibodies. As far as is known this study is the first class specific (IgG and IgA) evaluation of these antibodies, in patients with cervical anaplasia.

LEVELS OF SERUM IMMUNOGLOBULINS IN CERVICAL CARCINOMA

Since the introduction of the method of single radial immunodiffusion by Mancini et al in 1965 several studies have been made on the levels of immunoglobulins in a variety of diseases, malignant (Table 4.9) and non-malignant (Table 4.10).

Three studies have been reported which have examined levels of total immunoglobulin in cervical carcinoma (Table 4.11). Levels of IgA were elevated among patients in all three studies. Values of IgG were significantly elevated in the two studies which examined these levels (Vasudevan et al, 1971; Pleisnicar, 1970). The controls chosen in all three studies are not entirely satisfactory. In the study by Vasudevan et al, the control values used are ones established by Sehgal and Aikat (1970) in the same population but by a different modification of the Mancini method, and in a group with a heavy preponderance of males (107 males and 11 females). Within the same environment, levels of serum immunoglobulins vary with age, sex and social class (reviewed in McFarlane, 1973). In the other two studies two controls have not been matched for any attribute - in fact in the Pleisnicar (1970) study the average age of controls was 18 years younger than that of patients.

The lower levels of IgG and IgA in treated patients compared to untreated ones in the study done by Vasudevan et al imply that levels of total immunoglobulin may be of prognostic value. The value of levels of immunoglobulins

Table 4.9 Studies showing changes in level of IgG and IgA in malignancies other than carcinoma of the cervix

IMMUNOGLOBULIN	MALIGNANCY	REFERENCE
IgA		
Elevated levels		
	Cancer of larynx Cancer of breast	Dostalova et al (1970 and 1975)
	Cancer of epithelial surfaces	Waldman et al (1970)
	Hodgkin's disease	Dienstbier et al (1978)
	Oral cancers	Brown et al (1975)
IgG		
Elevated levels		
	Cancer of the kidney (Grawitz)	Dostalova et al (1975)
IgG and IgA		
Elevated levels		
	Cancer of the urinary bladder	Dostalova et al, (1975)
	Cancer of the bronchus	
	Primary cancer of the liver	Chew et al (1970)
	Kaposi sarcoma	Master et al (1970)
IgA		
Increasing levels with stage of disease		
	Cancer of breast Cancer of bronchus Cancer of larynx Cancer of bladder	Dostalova et al (1970)
IgG		
Increasing levels with stage of disease		
	Cancer of breast Cancer of bronchus	Dostalova et al (1976)

Table 4.10 Non-malignant conditions associated with changing levels of immunoglobulins

DISEASE	IMMUNOGLOBULIN AFFECTED	REFERENCE
Malaria	Elevated IgG Elevated IgM Elevated IgA	Reviewed in McFarlane, (1973)
Leishmaniasis	Mild elevation of IgA possible	
Filariasis	Mild elevation of IgM and IgG	
African trypanosomiasis	Elevation of IgM	
Helminthic infection	Elevation of IgM, IgG and IgA	
Schistosomiasis	Elevation of IgM, IgG and IgA	
Amoebiasis	Elevation of IgG, IgM and IgA	
Tuberculosis	Elevation of IgG, IgM and IgA - mostly IgA	
Syphilis, gonorrhoea and salmonella	Elevation of IgM and IgG	
Cholera	Elevation of IgM and IgA	
pneumoconiosis	Elevated IgA	Hagadorn and Burrel, (1968)
gout		Ball et al, (1969)
hepatic cirrhosis		Tomasi, (1968)
purpura		Trygstad and Steihm (1971)
bronchiectasis		South et al, (1967)
cystic fibrosis		
osteomyelitis		Waldman et al, (1970)

Table 4.11 Levels of IgG and IgA in patients with invasive carcinoma of the cervix - Findings in three studies

REFERENCE	STUDY GROUP	METHOD	RESULTS
Vasudevan et al (1971) (India)	Patients 24 prior to treatment 10 treated 8 months to 1½ years previously Controls None	SRID Mancini method as modified by McKelvey and Farhey Controls values established by Sehgal and Aikat (1970) have been used.	Significantly elevated levels of IgG and IgA in untreated patients. Levels of IgG and IgA similar to those of control values in treated patients.
Dent and Bienenstock (1970) (Canada)	Patients 57 Controls 28 healthy subjects from a fertility clinic	SRID Mancini technique	Significantly elevated levels of IgA in serum and cervico vaginal secretions in patients.
Pleisnicar (1970) (Sweden)	Patients 77 Controls 33 female blood donors.	SRID Mancini method as modified by McKelvey and Farhey	Significantly higher values of IgG in patients. Higher values of IgA in patients, but difference not significant.

as markers of occult tumor recurrence has not so far been studied.

In this study, levels of IgG and IgA in the first serum samples of patients with cervical anaplasia have been compared with those of controls matched for age, sex and ethnic origin. In addition levels in serial samples of serum have been tested with a view to examining their prognostic value.

BIOCHEMICAL MARKERS IN MALIGNANCY

A number of antigen systems have recently been investigated for their value as immunodiagnostic procedures in human cancers (Table 4.12). Of these up to present time, human choriogonadotrophic determination (HCG) in chorioncarcinoma has been the most successful immunodiagnostic method in reducing mortality from any one malignancy.

CEA was first described by Gold and Freedman in 1965. It is a glycoprotein of approximate molecular weight 200,000 which is found in the alimentary tract, liver and pancreas of fetuses between the second and sixth months of intrauterine life. With the development of sensitive radioimmunoassay, CEA has been detected in the serum or plasma of patients with a variety of tumors (Lo Gerfo et al, 1971), but the main problem with CEA as a marker of malignancy is that there is a high rate of false positives (Stevens et al, 1975). For this reason it has limited use as a diagnostic marker. Serial estimations however have proved to be of prognostic value in gastric cancer (Freeman et al, 1979) and colorectal cancer (reviewed in Neville and Cooper, 1976).

Elevated levels of CEA were first reported in patients with gynaecological malignancies in 1971 (Lo Gerfo et al, 1971), after which it was shown that CEA from cervical cancer and CEA from colonic cancer are immunologically similar (Goldberg et al, 1976).

Recent studies have also indicated the possible use of CEA as a marker of occult recurrence in cervical cancer (Di Saia et al, 1976; Khoo and Mackay, 1974; van Nagell et al, 1978). In the study by van Nagell et al (1978), serial estimations of CEA were done on 143 patients with carcinoma of the cervix (number of adenocarcinomas among them has not been stated). Forty-four of these patients developed tumor recurrence and 99 progressed well. Rising levels of CEA were observed in 29 of 44 (65%) patients who developed tumor recurrence and in only 1 of 99 (1%) who progressed well.

It has been suggested that in the immunodiagnosis of malignancy, several tests used concurrently may provide more discriminatory data than a single test (Herbermann, 1977). This concept has been extended in this study to the detection of tumor recurrence, and the possibility has been explored of several tests used in combination providing specific and sensitive information of occult recurrence. The markers that have been studied are a) membrane antibody to viral antigen (HSV-2), b) fetal antigen (CEA), and c) levels of total immunoglobulin (IgG and IgA).

Table 4.12 Some antigen systems of use in the immunodiagnosis of human cancer

TUMOR DERIVED PRODUCT	MALIGNANCY	REFERENCE
Carcinoembryonic antigen in plasma (CEA)	Colorectal carcinoma	Reviewed in Neville and Cooper (1976)
	Female genital cancers	
	Breast carcinoma	
	Bronchial carcinoma	
Human choriogonadotrophin (HCG) in plasma	Chorion carcinoma	
Alphafetoprotein (AFP) in plasma	Hepatomas and teratomas	Reviewed in Herberman (1977)
Thyrocalcitonin in plasma	Medullary carcinoma of the thyroid	
Fetal sulphoglycoprotein antigen (FSA) in gastric juice	Gastric cancer	
Ferritin in plasma	Breast carcinoma	

SPECULATION ON THE PREVENTION OF HSV-2 GENITAL HERPES AND THEREBY CERVICAL CARCINOMA BY VACCINATION

Melnick (1976) foresees that cervical carcinoma may be irradicated by the development of a suitable herpes simplex vaccine. Skinner et al (1977) have made the observation that more controls than patients with cervical anaplasia possess only HSV-1 specific antibody without HSV-2 specific antibody. They suggest that this inverted case control prevalence of HSV-1 specific antibody alone may be an indication that pre-pubertal exposure to HSV-1 portects from or modifies infection by HSV-2.

Nahmias et al (1978), have drawn an interesting analogy between the role of Turkey herpes virus (THV) in preventing Marek's disease and HSV-1 in preventing cervical carcinoma. Both whole virus and virus capsid vaccine of THV protects from the lymphoproliferative disease caused in chickens by MDV but not from infection caused by MDV (Purchase, 1976). These workers have proposed that previous HSV-1 infection may similarly protect from malignant change induced by HSV-2. Because HSV-1 infection is commonly acquired in early childhood, primary HSV-2 genital herpes (i.e. HSV-2 genital herpes in a patient with no prior exposure to HSV-1) is rare. However, if the hypothesis by Nahmias et al is correct, a greater proportion of patients with cervical anaplasia could be expected to possess only HSV-2 specific antobody in comparison with the proportion of women in the general population who have only HSV-2 specific antibody.

AIMS AND OBJECTIVES OF THIS STUDY

The primary aim of cancer research is to determine its cause and by so doing if possible to prevent it. Improved methods of early diagnosis which may reduce morbidity and mortality are two important objectives. Epidemiological studies may identify groups at high risk due to genetic, environmental, dietary, behavioural, occupational or infective reasons. Screening tests of high sensitivity and specificity are sought for the detection of pre-malignant and pre-clinical lesions. Therapeutic methods and tools are continuously assessed and reassessed. Laboratory tests are sought for monitoring treated patients to determine the effectiveness of treatment and to detect occult tumor recurrence. Lastly, methods of immunological control are sought by means of vaccination and immunotherapy. Thus the aims of the studies described in this thesis were:

1. To conduct a pilot study which tested the strength of the association between HSV-2 and cervical carcinoma in three countries - Sri Lanka, Malawi and Sudan - in which no studies have yet been conducted and to confirm this association which two previous studies conducted in the U.K. have demonstrated.

2. To study some of the relevant epidemiological characteristics of women with cervical anaplasia in these four countries.

3. To assess the effect that screening by Papanicolaou smear has had on the incidence rates and mortality rates from cervical carcinoma in England and Wales.

4. To study the occurrence of antibodies to membrane antigens (MA) of HSV-2 infected cells, virus capsid antigens (VCA) of HSV-2 infected cells, and HSV-1 and HSV-2 type specific antigen, in women with cervical anaplasia, controls matched for sex, age, ethnic origin and social class, and in other control groups such as patients with genital and extra-genital herpetic infection and patients with a variety of other malignancies. To examine the antibody profiles of the above groups of patients from the view-point a) of early diagnosis and prognosis, b) of the possible protection that previous HSV-1 infection may confer on subsequent infection by HSV-2 or subsequent malignant transformation by HSV-2, and c) of the nature of the association between HSV-2 and cervical carcinoma.

5. To study levels of serum IgG and IgA and carcinoembryonic antigen in patients with cervical anaplasia and to assess if these are of any prognostic significance.

Chapter 5

MATERIALS & METHODS

PATIENTS AND CONTROLS

PATIENTS AND CONTROLS

Table 4.1 contains the groups of patients and controls in the study. These included patients with cervical anaplasia (dysplasia carcinoma in situ and invasive carcinoma) from Britain (Groups 1-4), and patients with invasive carcinoma from Sri Lanka, Malawi and Sudan (Groups 5, 6 and 7). Controls for patients with cervical anaplasia were matched for age, sex, social class, and ethnic origin. Other control groups included patients with genital and extra-genital herpetic lesions (Groups 11 and 12) and patients with malignancies other than cervical carcinoma. The patients with malignancies other than cervical carcinoma were divided into three broad categories (Groups 8, 9 and 10). Group 8 consisted of patients with genital malignancies other than squamous cervical carcinoma; group 9 of patients with non-genital squamous carcinoma, and group 10 of patients with extra-genital non-squamous malignancies.

The majority of sera from British patients were collected personally at St. Thomas' Hospital, London. The names of patients having abnormal smears was obtained from the cervical cytology report book in the department of cytology. These patients were met at the gynaecology clinic by tracing their names in the consultant's appointment book at the gynaecology clinics and seen at the wards by following the admissions lists to the wards. Patients who were treated by radiotherapy were met at the combined gynaecology and radiotherapy clinic. The names of patients with malignancies other than cervical carcinoma were obtained from the surgical pathology report book. There were both caucasian and negroid

(mainly West Indian) patients among those seen at St. Thomas', and they are collectively referred to in this thesis as British patients.

PATIENTS WITH DYSPLASIA OF THE UTERINE CERVIX (GROUP 1, TABLE 4.1)

These were patients seen at the gynaecology clinic at St. Thomas' Hospital. The diagnosis of dysplasia was made on cervical cytology alone and consisted of patients with a grade 3A or 3B Papanicolaou (Pap) smear (see page 76 for grading of Pap smears). Serial samples of serum were collected from 30 of these patients when they presented for repeat smear which was approximately once in every six months. The epidemiological data of a further 22 patients from whom it was not possible to obtain serial samples of serum was made use of for analysis of data according to age, social class and ethnic origin (Part I of Results).

PATIENTS WITH CARCINOMA IN SITU OF THE UTERINE CERVIX (GROUP 2, TABLE 4.1)

These were also patients seen at the gynaecology clinic at St. Thomas' Hospital. The diagnosis of carcinoma *in situ* was made on histological examination of cone biopsy material following the detection of a grade 4 Pap smear. Serial samples of serum were collected from 29 of these patients, of which the first serum was in each case collected prior to cone biopsy, and subsequent samples when patient presented for follow up at the clinic. The epidemiological data of

15 patients from whom serial samples could not be collected were used in epidemiological analysis.

PATIENTS WITH INVASIVE SQUAMOUS CARCINOMA OF THE UTERINE CERVIX

Sera from patients with invasive carcinoma were obtained from four countries - Britain, Sri Lanka, Malawi and Sudan. They had been staged according to the recommendation of the Federation of International Obstetricians and Gynaecologists (FIGO, 1965) (see page 78).

BRITISH PATIENTS WITH INVASIVE SQUAMOUS CARCINOMA OF THE UTERINE CERVIX (GROUPS 3 AND 4, TABLE 4.1).

All British patients with invasive carcinoma were ones treated at St. Thomas' Hospital. The diagnosis was made by histological examination of biopsy material after:

a) Patient presented with symptoms suggestive of cervical carcinoma, e.g. vaginal discharge or post coital bleeding, or

b) following the detection of a grade 4 Pap smear.

In 29 patients, the first serum sample was obtained prior to treatment (Group 3, Table 4.1). The second serum sample was collected at the termination of treatment and thereafter at follow up visits to the clinic.

In 41 patients (Group 4, Table 4.1), the first serum sample was collected 1-12 years after treatment. These were patients being followed up at the combined radiotherapy and gynaecology clinics. Thereafter, serial samples of serum were collected from 30 patients - at each subsequent visit

to the clinic. The epidemiological data from the 11 patients from whom no serial samples could be obtained was made use of for appropriate analysis.

SRI LANKAN PATIENTS WITH INVASIVE SQUAMOUS CARCINOMA OF THE UTERINE CERVIX (Group 5, Table 4.1)

These were patients from Ward 8 of the Cancer Institute at Maharagama Sri Lanka, who had been referred from hospitals in all parts of the island. The diagnosis of squamous carcinoma of the cervix was made on histological examination of biopsy material following clinical examination for symptoms suggestive of the tumor. Routine cervical cytology is not yet carried out in Sri Lanka. The first serum samples from all Sri Lankan patients was obtained prior to treatment. The initial few were collected personally on a visit to Sri Lanka and thereafter both pre-treatment samples from fresh cases as well as serial samples, were collected by Dr. Lorraine Senarath, the house officer in charge of Ward 8. It was not possible to obtain serial samples for as long a period of follow up as for British patients because some of these patients were referred to their local hospitals for follow up and others defaulted attendance at the follow up clinic at the cancer institute. Among the Sri Lankan patients there were both Sinhalese and Tamils.

MALAWIAN PATIENTS WITH INVASIVE SQUAMOUS CARCINOMA OF THE UTERINE CERVIX (GROUP 6, TABLE 4.1)

Sera from these patients was arranged for by Professor

M.S.R. Hutt of the Geographical Pathology Unit at St. Thomas' Hospital and were collected and shipped by Dr. J. Chiphangwi of the Queen Elizabeth Central Hospital at Blantyre, Malawi. Pre-treatment samples of serum were sent from 27 patients with invasive squamous carcinoma diagnosed by histological examination of biopsy material from the tumor. Post-treatment samples of serum were obtained only from four of these patients. These patients had been drawn from the area around Blantyre and were all Malawian.

SUDANESE PATIENTS WITH INVASIVE SQUAMOUS CARCINOMA OF THE UTERINE CERVIX (GROUP 7, TABLE 4.1)

Pre-treatment samples of serum from 27 patients with histologically confirmed squamous carcinoma of the cervix was provided by Dr. M.O.A. Malik. These were control sera being used by him on a study on nasopharyngeal carcinoma in Sudanese in collaboration with the department of pathology and virology at St. Thomas' Hospital. For this reason it was not possible to obtain either serial samples of serum from patients, or sera from matched controls.

CONTROLS FOR PATIENTS WITH CERVICAL ANAPLASIA

Controls for British patients with cervical anaplasia

A pool of 133 control sera were collected at the gynaecology clinic at St. Thomas' Hospital. These were all patients who presented with complaints such as menorrhagia, dysmenorrhoea, subfertility, etc. on whom a recent normal smear report was available. They were later matched with

patients with cervical anaplasia according to age, ethnic origin and social class.

Controls for Sri Lankan patients with invasive carcinoma

A pool of 44 Sri Lankan control sera were collected personally on a visit to Sri Lanka at the gynaecology clinic at the De Soysa maternity home at Colombo 8. Since cervical smear reports were not available on these patients, patients who had apparently healthy cervices on speculum examination were chosen. These were patients who presented for symptoms unrelated to cervical carcinoma. Of this pool, of 44 controls, only 17 could be matched with Sri Lankan patients with invasive carcinoma on the basis of age, social class and ethnic origin.

QUESTIONNAIRE FOR PATIENTS WITH CERVICAL ANAPLASIA AND FOR CONTROLS

A specimen of the format for personal history and clinical history of patients with cervical anaplasia and of controls is given in Appendix A. Precise information was not available on the Sudanese patients.

PATIENTS WITH OTHER GENITAL MALIGNANCIES (GROUP 8, TABLE 4.1)

Pre-treatment samples of serum were collected from 33 patients with other genital malignancies who were treated in the gynaecology wards at St. Thomas' Hospital. They consisted of the following groups of patients:

Adenocarcinoma of the cervix	7
Leiomyosarcoma of the uterus	1
Endometrial carcinoma	17
Adenocarcinoma of the ovary	3
Teratoma of the ovary	1
Cystadeno carcinoma of the ovary	1
Squamous carcinoma of the vulva	3

PATIENTS WITH NON-GENITAL SQUAMOUS CARCINOMA (GROUP 9, TABLE 4.1)

Pre-treatment samples of serum were collected from 24 patients with squamous carcinoma at different sites of the body. Samples from three patients with squamous carcinoma of the larynx were provided by Dr. K.E.K. Rowson of the Institute of Laryngology and Otology at Gray's Inn Road and samples from four patients with squamous carcinoma of the bronchus were provided by Dr. K.J. Randall of the Orpington Hospital at Kent. The sites of occurrence of squamous carcinoma in these patients were as follows:

Squamous carcinoma	of the bronchus	6
	of the lung	3
	of the larynx	7
	of the oral cavity	2
	of the oesophagus	2
	of the ano-rectal junction	1
Secondary Sq. Ca. metastasis in cervical nodes, primary not known		3

PATIENTS WITH EXTRA-GENITAL NON-SQUAMOUS MALIGNANCIES (GROUP 10, TABLE 4.1)

Pre-treatment samples of serum from 24 such patients were collected. Thirteen pre-treatment samples were provided by Dr. K.J. Randall. The malignancies in these patients were as follows:

Adeno carcinoma of the colon and rectum	11
Invasive carcinoma of the breast	8
Adeno carcinoma of the prostate	4
Adeno carcinoma of the bile duct, testis and pancreas (one each)	3
Anaplastic carcinoma of the stomach	4
Poorly differentiated malignancy of the bladder	5
Malignancies of the kidney	2

PATIENTS WITH GENITAL HERPES (GROUP 11, TABLE 4.1)

Serial samples of serum from more than 16 patients with genital herpes were provided by Dr. B.F. Carrol of the genito-urinary department at St. Thomas' Hospital. Of these, 16 patients were chosen for the study in whom there was virological confirmation that the infecting virus was HSV-2. Typing of the viral isolate was on the basis of cytopathic effect (CPE) on human embryo lung cells and pock size on the choriallantoic membrane (CAM) of eggs. The first serum samples from all these patients was collected within two months of their first clinical attack of genital herpes. Thereafter, attempts were made at virus isolation from the cervix and vulva at subsequent visits to the clinic despite there being no apparent clinical lesions.

PATIENTS WITH EXTRA-GENITAL HERPES (GROUP 12, TABLE 4.1)

Serial samples of serum were collected from 16 patients presenting with extra-genital herpetic lesions to the department of Virology at St. Thomas' Hospital. Following initial diagnosis by elctronmicroscopy the infecting virus was confirmed as being HSV-1 by the appearance of CPE on human embryo lung cells and pock size on CAMs.

SOCIAL CLASS GRADING

British patients were divided into five social classes using the system of the U.K. Registrar General (Classification of occupations, 1970). The husband's job was used as the index for classifying married women, divorcees and widows, the father's job to classify unmarried women who were not employed, and unmarried women who were employed were classified according to their own jobs.

Sri Lankan patients were classified into three social class groups: low, middle and upper. This division was guided by job status and income.

Malawian and Sudanese patients were classified into three groups: low, middle and upper, by Dr. Chipangwi and Dr. M.O.A. Malik respectively.

Collection and storage of sera.

Twenty millilitres of blood was drawn from the cubital vein. It was allowed to clot at 37°C in a water bath for 15 minutes, left overnight at 4°C and the serum separated by centrifugation in a bench centrifuge. Serum was aliquoted and stored at -20°C.

Table 5.1 Country of origin, patients and controls

	DIAGNOSIS	NUMBER	SERIAL SA's	COUNTRY
1	Dysplasia	30 (22)	Yes	Britain
	Controls	27	-	
2	Ca *in situ*	29 (15)	Yes	Britain
	Controls	29	-	
3	Untreated invasive Ca.	29	Yes	Britain
	Controls	22	-	
4	Treated invasive Ca.	30 (11)	Yes	Britain
	Controls	15	-	
5	Invasive carcinoma	32	from 29	Sri Lanka
	Controls	17	-	
6	Invasive carcinoma	27	from 4	Malawi
	Controls	18	-	
7	Invasive carcinoma	27	-	Sudan
	Controls	-	-	
8	Other genital malignancies	33	-	Britain
9	Non-genital squamous carcinoma	18	-	Britain
10	Extra-genital non-sq. Ca.	37	-	Britain
11	Genital herpes	16	Yes	Britain
12	Extra-genital herpes	16	Yes	Britain

( ) no. of patients from whom it was not possible to collect serial samples

METHOD 1

IgG AND IgA MEMBRANE ANTIBODY ASSAY ON HSV-2 AND HSV-1 INFECTED CELLS.

IgG AND IgA MEMBRANE ANTIBODY ASSAY ON HSV-2 INFECTED CELLS

All equipment and materials employed in the membrane fluorescent assay are described in Appendix B.

CELLS

Rabbit kidney cells (RK13) (B17) were grown in polystyrene disposable bottles of surface area 150 cm^2. Both the growth medium (B18) and maintenance medium (B19) contained minimal essential medium supplemented with fetal calf serum, glutamine and sodium bicarbonate. The quantity of medium used was 50 ml per 150 cm^2 bottle. When the cells were confluent, they were split one bottle to three bottles using a solution of trypsin and versene (B22). They were grown at 37^{o}C and inoculated with virus on the third day after splitting by which time the cell sheet was confluent.

VIRUS

The Bry strain of HSV-2 virus was used (B30A) which was grown on RK13 cells and titred on the chorioallantoic membrane of fertile incubated eggs (Appendix C).

INFECTING CELLS WITH VIRUS

A virus inoculum of 1 p.f.u. per cell was used and left to adsorb at room temperature for one hour. Thereafter the virus inoculum was discarded and 50 ml of maintenance medium added. The infected cells were incubated at 37^{o}C till there was a 50-75% cytopathic effect (CPE) which usually developed in 15-17 hours.

CONTROL CELLS

RK13 cells were mock inoculated with sterile phosphate buffered saline (PBS - B25) which was discarded at the end of one hour after which maintenance medium was added and the cells incubated for the same length of time as the infected cells.

Harvesting of infected and control cells

Both control and infected cells were harvested into the maintenance medium by introducing into the bottle glass beads of diameter 3-4 mm, and shaking gently with the bottle closed and held horizontally, till all the cells had been detached from the bottle surface.

Washing of cells

The infected cells and control cells were separately pooled into two or more centrifuge bottles and centrifuged (B13) for 10 minutes at 1000 x g. The supernate was discarded and the cells washed twice in freshly prepared non-sterile PBS.

Distribution of cells into microtitre plates

The cells were resuspended in freshly made PBS and distributed into the wells of U-shaped microtitre plates (B2) such that wach well received approximately 2-5 x 10^5 cells, i.e.

Each bottle yielded 14-16 x 10^6 cells (approximately)

Each microtitre well was filled with 0.2 ml of cell suspension.

Therefore $\frac{16 \times 10^6}{5 \times 10^5} \times 0.2 = 6.6$ ml

i.e. the cells from one bottle required to be suspended in 6.6 ml of PBS such that 0.2 ml would contain approximately 5×10^5 cells. Thus the cells harvested from one bottle was sufficient for approximately 32 wells. Control cells were distributed into control wells and infected cells into test wells as indicated in Figures 4.1 and 4.2. The plates were spun at 1000 x g for 5 minutes and the supernate removed with a finely drawn pasteur pipette which was attached to a vacuum pump.

SERUM DILUTIONS

All sera were tested under code. All sera were heat inactivated at 56°C for half an hour. This reduced the tendency of some sera to cause clumping of cells. Serial samples of serum from a single patient were always tested the same day. Dilutions were made in freshly made PBS in microtitre plates. The starting dilution in the IgG membrane fluorescence assay was 1:8 and in the IgA assay 1:4.

A hand multidilutor (B6) was used with 0.05 diluters and employing two rows of wells for each serum. 0.05 ml disposable pipettes (B3) were used to distribute PBS to the plates and Finn pipette (B4) and disposable tips (B5) to transfer aliquots of serum to the starting well. By pooling similar serum dilutions in both rows. 0.1 ml of each serum dilution was added to each well containing the infected or

control cells using a Finn pipette and disposable tips. One tip was used per serum starting at the highest dilution. As experience was gained, the dilutions indicated in Figure 4.1 were found to be the most convenient for use in the IgG assay and those in Figure 4.2 for the IgA assay. Any serum which had no detectable antibody at a 1:64 dilution or appeared to be of a higher titre than 1:1024 in the IgG assay was retitred using the appropriate dilution range. Similarly, in the IgA assay, sera which appeared to be of a higher titre than 1:64 were retitred. The serum dilution added to the control cells was always 1:8 in the IgG assay and 1:4 in the IgA assay in order to detect non-specific fluorescence.

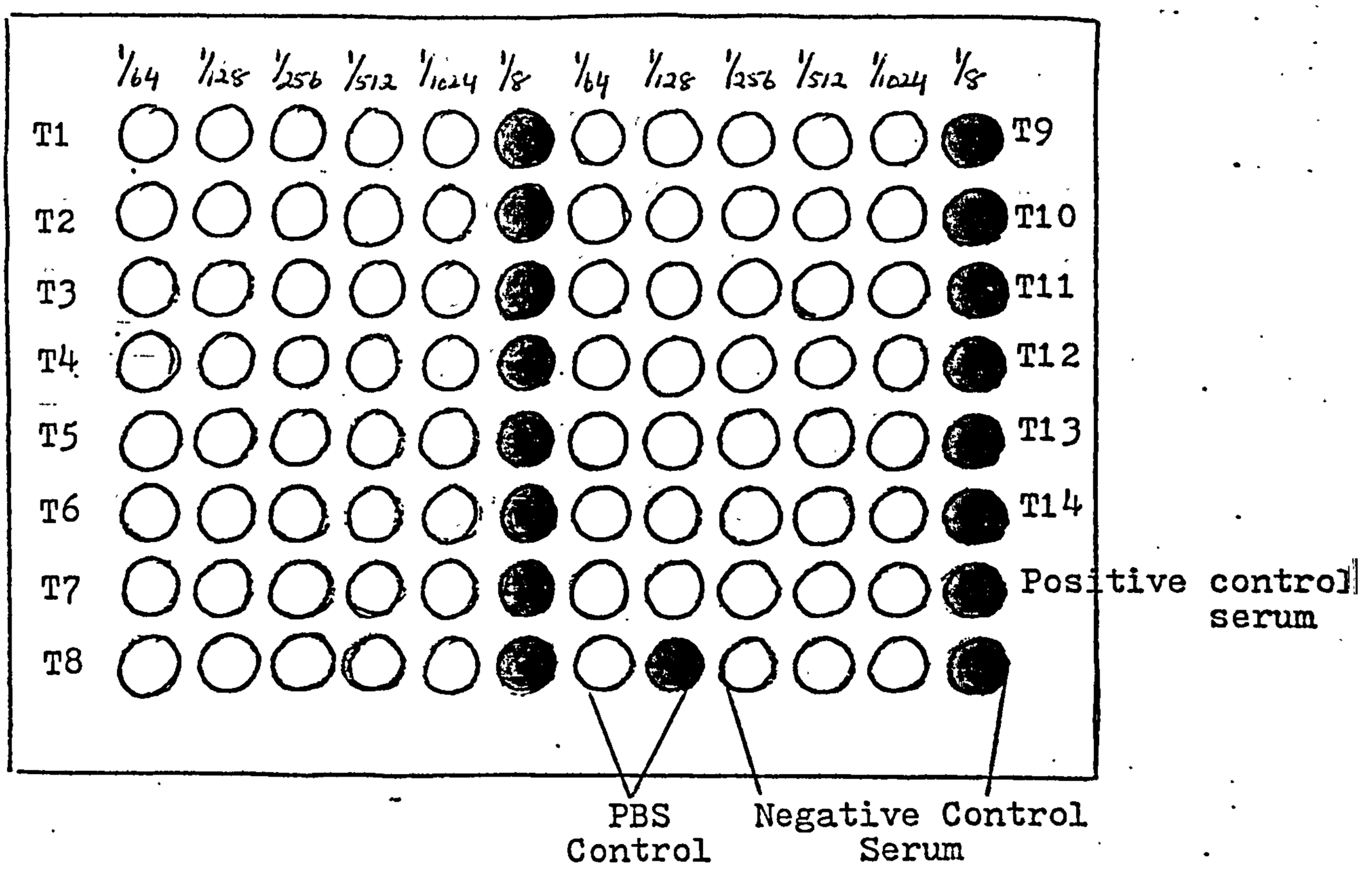
CONTROLS

The same positive control serum and negative control serum was used in all tests. These sera were stored in aliquots of 0.2 ml. Thus a fresh aliquot was used in each test. In addition, a PBS control was used in each test. It was thus possible to test 30 sera per day (2 plates).

Incubation of serum with cells.

Once the serum dilutions were added to the cells, the plate was placed very lightly on a rotor mixer (B11) so that good mixing of cells and serum was ensured. The plates were incubated for one hour in an airtight moisturized plastic sandwich box.

Figure 4.1 Template for IgG membrane fluorescence assay.

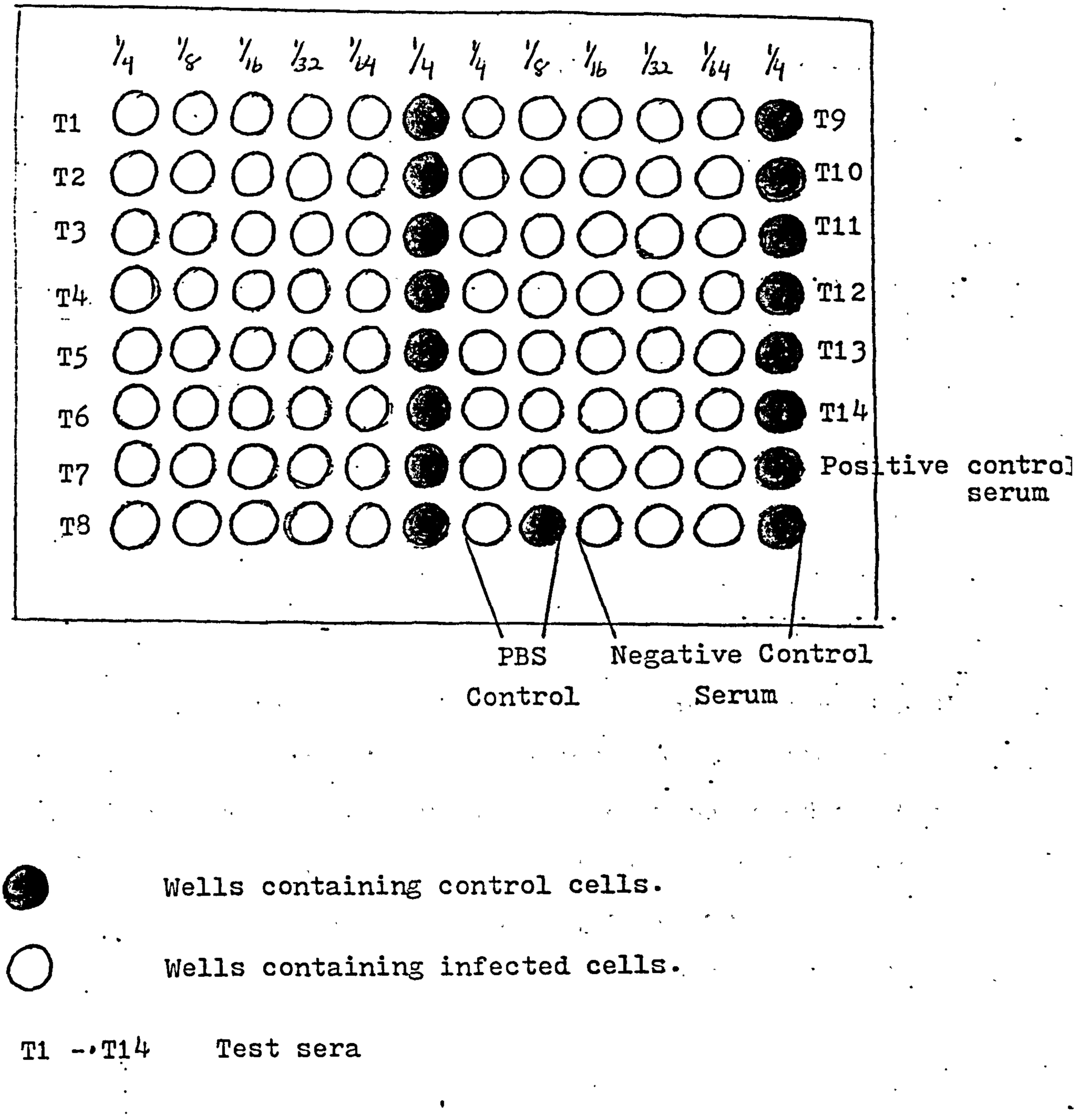


● Wells containing control cells.

○ Wells containing infected cells.

T1 - T14 Test sera

Figure 4.2 Template for IgA membrane fluorescence assay.



● Wells containing control cells.

○ Wells containing infected cells.

T1 - T14 Test sera

Washing of cells

At the end of one hour, each well was topped with PBS with a pasteur pipette and centrifuged at 100 x g for 5 minutes. The supernate was sucked out as explained above. The cells were agitated very briefly by placing the plates on the rotor mixer and the washing procedures repeated twice more. The supernate was carefully and completely sucked out at the end of the third wash.

Adding of conjugate

Rabbit antihuman IgG fluorescein isothiocyanate was used in the IgG membrane fluorescence assay and rabbit anti-human IgA fluorescein isothiocyanate in the IgA membrane fluorescence assay (B31).

The conjugate had been previously absorbed (Appendix E) with pig liver powder (Appendix D) and control cells, and the optimum working dilution decided on by means of checker board titration. 0.05 ml of conjugate at the optimum working dilution was added per well. The cells and conjugate were mixed well on the rotor mixer and incubated for one hour at 37°C as described above.

Washing of cells

As described above. After the third wask the supernate was withdrawn as completely as possible using a finely drawn pasteur pipette.

Resuspension of cells in glycerol-PBS

A 50% solution of glycerol (B27) in PBS was prepared fresh and a minute drop added to each well employing a finely drawn pasteur pipette. The cells and glycerol-PBS were mixed well by placing the plate lightly on the rotor mixer.

Spotting the resuspended cells on microscope slides

Employing a Finn pipette and a disposable tip per serum, cells in each well were spotted on glass microscope slides (B7) beginning with the well containing the control cells and working through from the well with the highest serum dilution.

One slide was sufficient for the cells reacting with one serum (6 spots). Round glass coverslips (B9) were dropped over each spot.

Storing of slides

The slides were placed in a cardboard slide holder and stored at -20^{o}C for 24-118 hours before reading.

Reading of slides

The slides were read using a fluorescent microscope with an epifluorescence condenser and phase contrast (B14).

Determination of end-point

The positve control serum used in the IgG assay repeatedly

titred at 1:128 or 1:256. This slide was examined after the slides containing PBS controls and the negative control serum, and the proportion of fluorescent cells at the 1:128 dilution noted. In each test serum the end point was the reciprocal of the serum dilution which gave a similar proportion of fluorescent cells of the same degree of brightness as observed in the 1:128 dilution of the positive control serum. In this manner the best possible standardization was achieved not only between the sera in one assay but also of sera tested on separate days.

Nature of membrane fluorescence

Only cells showing clear membrane fluorescence such as shown in Figure 4.3 were considered, e.g. dead cells as showing in Figure 4.4 in which the whole cell takes up fluorescent stain was not considered. When the site of fluorescence was in doubt it was helpful to observe them under phase contrast together with fluorescence, Figure 4.5. Slides chosen randomly were sometimes checked by an independent reader.

Non-specific fluorescence

The sera which showed non-specific fluorescence on control cells were re-tested after prior absorption with control cells (Appendix F).

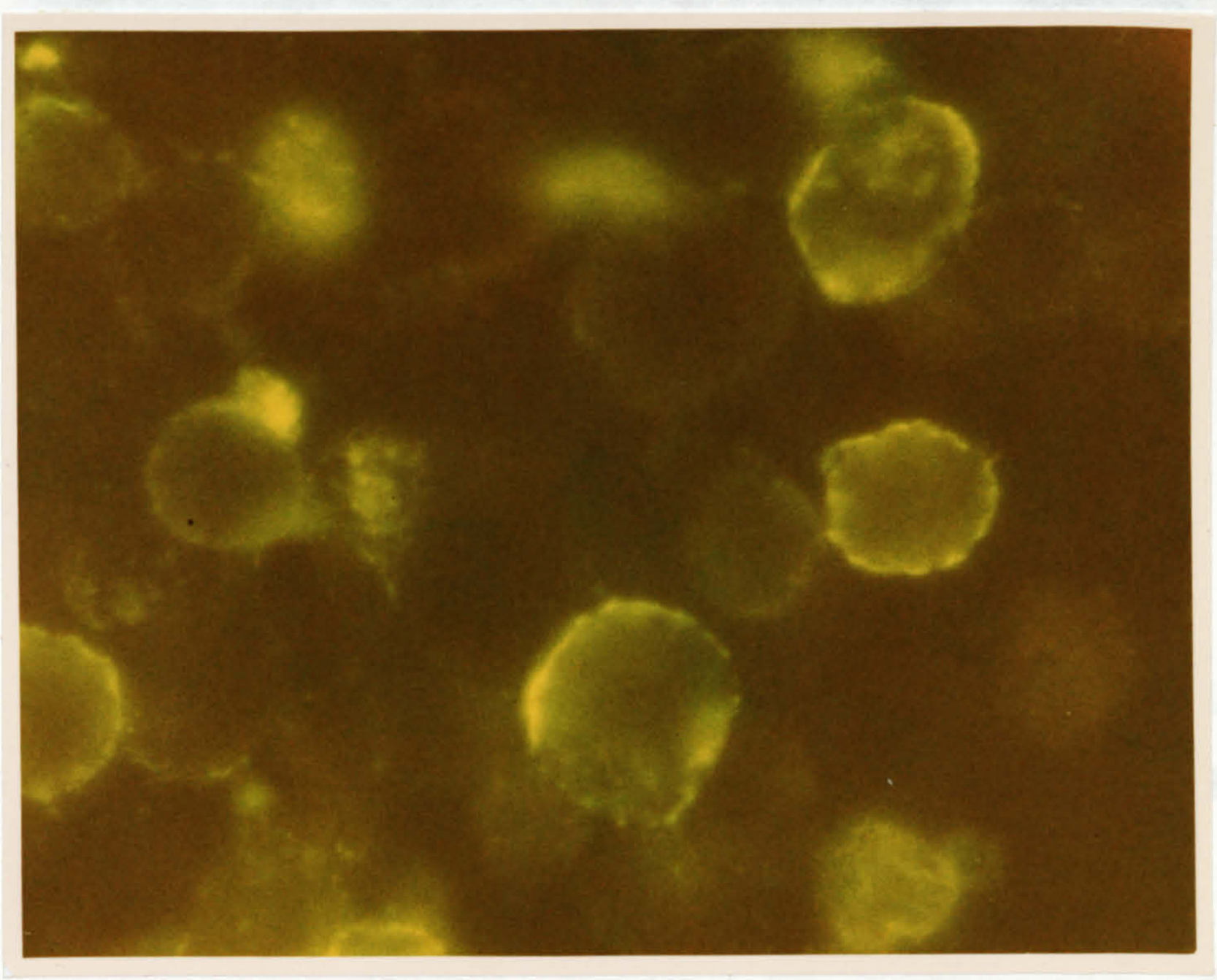


Figure 4.3 Rabbit kidney cells infected with HSV-2 showing membrane fluorescence.

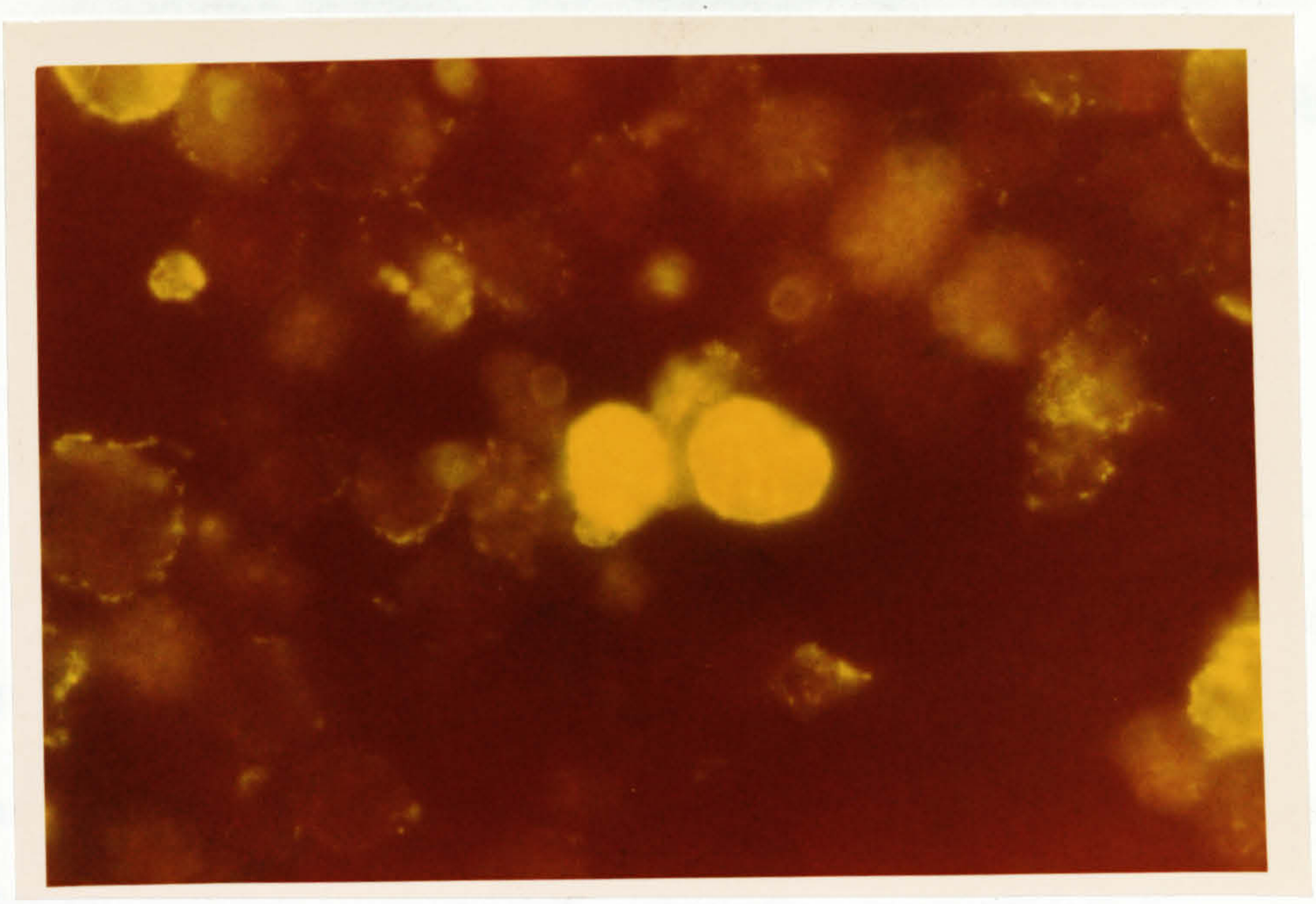


Figure 4.4 Appearance of dead HSV-2 infected rabbit kidney cells in the membrane fluorescence assay.

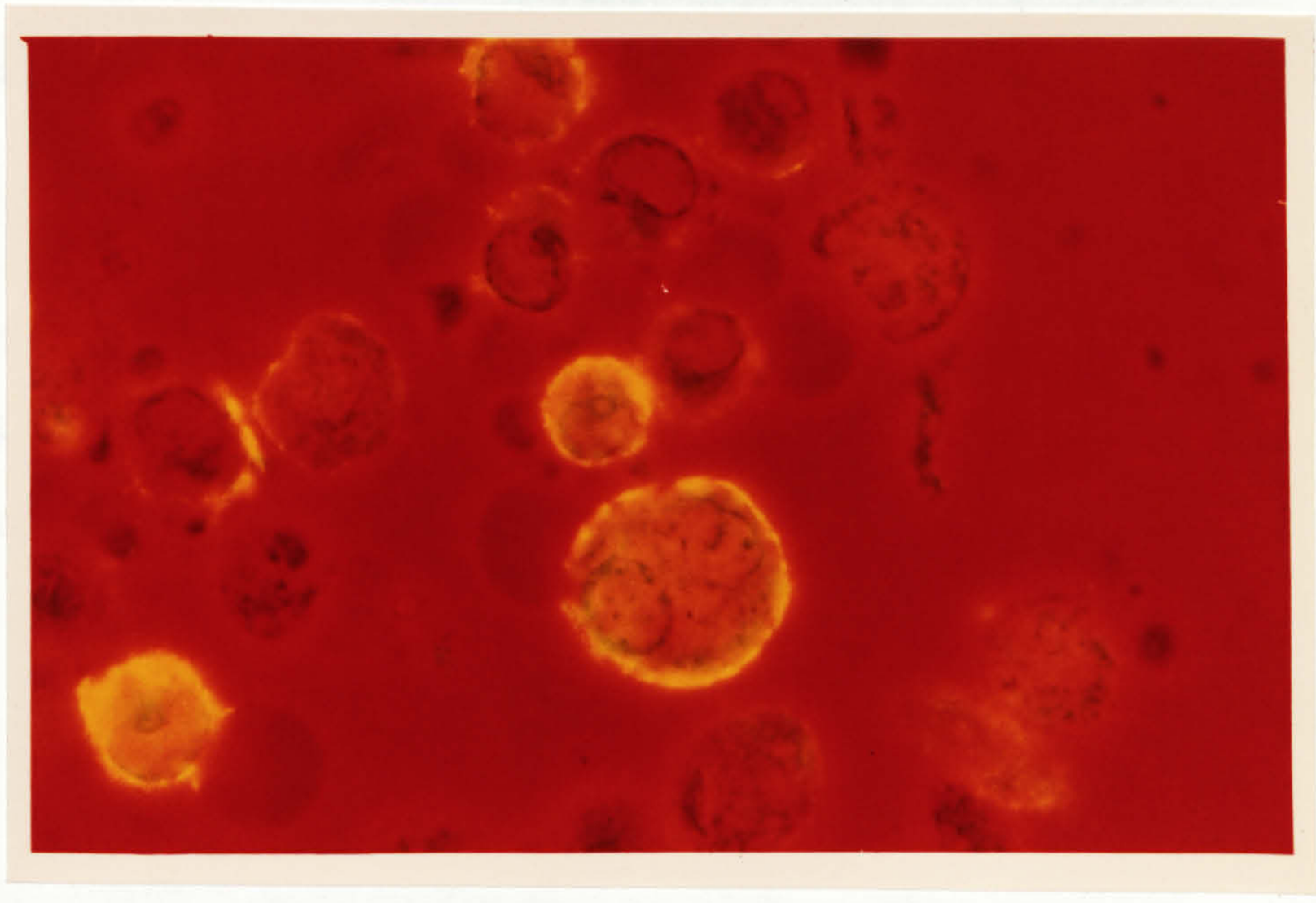


Figure 4.5 Rabbit kidney cells showing membrane fluorescence, observed with phase contrast and red filter.

The membrane fluorescence assay for IgG and IgA antibody to the membrane antigens of HSV-1 infected cells

All the equipment and materials employed in this assay are described in Appendix B.

The method was the same as described above for IgG and IgA antibody to membrane antigens of HSV-2 infected cells except for the following:

1. Virus - the HFEM strain of HSV-1 virus was used (B30B)
2. Concentration of infected cells - On distributing infected cells into microtitre wells, a slightly higher concentration of cells was used per well as the HSV-1 infected cells tended to disintegrate more rapidly than the HSV-2 infected cells. Each well received approximately 6×10^6 cells, i.e. each bottle yielded approximately 16×10^6 cells.

Each microtitre well was filled with 0.2 ml of cell suspension.

Therefore $\frac{16 \times 10^6}{6 \times 10^5} \times 0.2 = 4.6$ ml (approximately)

i.e. the cells from one bottle required to be suspended in 4.6 - 5 ml of PBS. Thus the cells harvested from one 150 cm^2 bottle was sufficient for approximately 24 wells.

METHOD 2

FLUORESCENT ASSAY FOR IgA ANTIBODIES TO VIRUS CAPSID ANTIGEN OF HSV-2 INFECTED CELLS

FLUORESCENT ASSAY FOR IgA ANTIBODIES TO VIRUS CAPSID ANTIGEN OF HSV-2 INFECTED CELLS

The equipment and materials used in this assay are described in Appendix B.

CELLS

Rabbit kidney cells (RK13) cells (B17) were grown in polystyrene bottles (B1) of surface area 150 cm^2 using growth medium (B18). On the third or fourth morning after splitting the cells, a confluent cell layer was obtained. They were then infected with virus.

VIRUS

The Bry strain of HSV-2 virus was used (B29A). A virus inoculum of 1 p.f.u./cell was used. The virus was allowed to adsorb for one hour at room temperature (RT) after which it was discarded, and 50 ml of maintenance medium (B19) added to the bottles. The infected cells were incubated at 37°C for nine hours.

CONTROL CELLS

Control cells were mock inoculated with PBS and incubated for the same length of time.

Harvesting and washing of cells

The maintenance medium was discarded and 20-30 ml of ice cold veronal buffer (B26) (VB) and a few glass beads of

diameter 3-4 mm were added to each bottle. The cells were gently detached from the surface of the bottle by shaking the bottle held in a horizontal position. The infected and control cells were pooled separately into centrifuge bottles and centrifuged at $4^{o}C$ at 1000 x g for 10 minutes. The washing process with ice cold VB was repeated twice more and the supernate discarded. The cells were suspended in 20 ml of VB per 150 cm^2 bottle, mixed well using a 10 ml pipette in order to break up clumps of cells and counted in a Neubauer counting chamber. The cells were diluted further with VB to give a cell concentration of 2.5×10^5 cells/ml.

Placing of cell suspension on slides

0,05 ml of cell suspension was dropped in each spot of multispot microscope slides (B8) using a disposable pipette (B3).

Drying of slides

The slides were allowed to air dry overnight at room temperature (RT).

Acetone fixation

The next morning the dry slides were placed in coplin jars, washed briefly with ice cold acetone which was discarded and left to fix for 20 minutes in fresh ice cold acetone. During this 20 minutes the coplin jars were kept at $4^{o}C$. The acetone was discarded and the slides allowed to air dry.

Storage of slides

The slides were wrapped in parafilm and stored at -70°C in cardboard boxes.

They were used within two weeks of preparing. Beyond this period the fluorescence obtained on them progressively faded.

Serological procedure

The slides were placed on a plastic mesh work frame which fitted into the lid of a sandwich box and allowed to come to RT.

Serum dilutions

The sera were thawed and heat inactivated (56°C for half an hour). Sera were tested under code. Serial samples of sera from one patient were tested in the same assay. Approxiamtely 41 sera were tested in one day. Two-fold serum dilutions were made in microtitre plates using a hand multidilutor (B6). 0.025 ml of each serum dilution was placed on each spot using a Finn pipette and a disaposable tip per serum, beginning at the highest dilution.

Controls

The same positive control serum and a negative control serum were used in all tests. In addition, PBS controls were set up both on infected and control cells.

Incubation

The sandwich box was moisturized, closed and incubated for one hour at 37°C.

Washing

At the end of one hour each slide was briefly rinsed with VB using a drop bottle and placed in coplin jars containing ice cold VB. The coplin jars were placed on a shaker at RT and the slides allowed to wash while the buffer was being gently agitated. Washing proceeded for half an hour, replacing with fresh buffer every 10 minutes. The slides were drained, gently mopped with tissue, replaced on the plastic mesh in the sandwich box lids and allowed to air dry. When completely dry the conjugate was added to each spot.

Conjugate

Rabbit anti-human IgA conjugate (B31) was used which had been previously absorbed (Appendix E) with pig liver powder (Appendix D) and control cells. The optimum working dilution of the conjugate had been previously determined by checker board titration . 0.25 ml of conjugate of the working dilution was added to each spot and incubated at 37°C for one hour in the air-tight moisturized box.

Washing

The slides were washed as described above.

Mounting of slides

The slides were mounted in 90% PBS glycerol (B27) using rectangular cover slips (B10). They were placed in cardboard slide holders and stored at -20°C and read within 48 hours.

Reading slides

The positive serum titred at 1:32 or 1:64 in each test. The end point of the test serum was the one in which clear cytoplasmic or perinuclear fluorescence was observed similar in intensity to that of the end-point in the positive serum.

METHOD 3

HSV-1 AND HSV-2 SPECIFIC ANTIBODY BY ENZYME LINKED IMMUNOABSORBENT ASSAY (ELISA)

ENZYME LINKED IMMUNOABSORBENT ASSAY (ELISA) FOR HSV-1 AND HSV-2 SPECIFIC ANTIBODY

These tests were conducted by Dr. B.F. Vestergaard of the Department of Clinical Virology, Institute of Medical Microbiology, University of Copenhagen, Denmark (Grauballe and Vestergaard, 1977; Vestergaard et al, 1977).

ANTIGEN

HSV-2 type specific antigen

Crude antigen was prepared from Triton X-100 solubilised HSV-2 infected rabbit cornea cells. The solubilised HSV-2 proteins were purified by ion exchange chromatography. The chromatographically purified glycoprotein preparation designated fraction 1 (Vestergaard and Grauballe, 1975) was next passed through a cyanogen-bromide activated Sepharose 4B column coupled previously with purified immunoglobulin of rabbit anti HSV-1 anti serum. The fractions which contained HSV-2 specific antigen were identified by screening by ELISA against a pool of HSV-1 antisera from children aged 6-11 years, and a pool of sera from patients with virologically confirmed HSV-2 infection. The fractions obtained immediately after the void volumn contained HSV-2 type specific antigens exclusively and were pooled and used in ELISA.

HSV-1 type specific antigen

HSV-1 type specific antigen was prepared in the same manner employing HSV-1 infected rabbit cornea cells. Sepharose

B was coupled with purified immunoglobulin from rabbit serum raised against HSV-2.

General virus antigen

Fraction I glycoproteins obtained following purification by ion exchange chromatography was used as general virus antigen.

Control antigen

Triton-X solubilised, uninfected rabbit cornea cells were purified by ion-exchange chromatography and the fraction I glycoproteins used as control antigens.

VIRUS

HSV-1 strain F

HSV-2 strain G

SERA

Test sera were tested at a dilution of 1:100.

The positive reference serum used against general virus antigen as a control for estimating antibody to type common antigen gave an optical density (OD) value of 70. The positive reference serum used against HSV-1 type specific antigen gave an OD value of 20, and that used against HSV-2 type specific antigen gave an OD value of 30.

Test sera which gave an OD value of greater than or equal to 10 were considered definite positives. Values

between 6 and 10 were considered as possible positives.

METHOD

Microtitre plates were used (Cooke M220-25A). The plates were pre-coated with 100 µl per well of 0.1% bovine albumin (Fraction V, Sigma no. A-4503) in distilled water. The bovine albumin solution was allowed to dry out at room temperature. 100 µl of 0.25% glutaraldehyde (25% glutaraldehyde, Fluka AG pract 49630) in PBS adjusted to pH 7.0 with NaOH added to each well. After 30 minutes at room temperature, the wells were emptied and washed three times in 150 µl of distilled water. 100 µl of a 1:200 dilution of antigen was then added to each well and allowed to dry out at room temperature followed by washing three times as described above. Test sera and peroxidase conjugated rabbit anti-human IgG (P-1090, DAKO Immunoglobulins, Copenhagen, Denmark) were diluted in a 0.5% bovine albumin with 0.05% Tween 20 (Art 822184, Merck) in PBS. 100 µl of test serum dilutions were added to each well and incubated for one hour at 37°C followed by washing three times with 150 µl of 0.05% Tween 20 in PBS. 100 µl of the enzyme conjugate diluted 1:200 was then added, followed by incubation and washing as described above. 55 mg of 1:2 phenylendiamindihydrochloride (Fluka AG, puris) was dissolved in 100 ml of an 0.04 M Tris-HCl buffer pH 7.6 with 0.9% NaCl. Just before use, 30 µl of 30% H_2O_2 was added and 100 µl of the reaction mixture was placed in each well. The reaction proceeded for 5 minutes at room temperature and was stopped by the addition of 50 µl of 0.1 N H_2SO_4. The contents of each well were transferred to 0.5 ml if 0.1 N H_2SO_4 and the OD was read in a Zeiss spectrophotometer at 450 nm.

METHOD 4

HSV-1 AND HSV-2 SPECIFIC ANTI-BODY BY COMPLEMENT FIXATION TES

DETECTION OF HSV-1 AND HSV-2 SPECIFIC ANTIBODY BY COMPLEMENT FIXATION TEST (SKINNER, HARTLEY AND WHITNEY, 1976)

Some of the sera used in this study were tested for HSV-1 and HSV-2 specific antibody using the above test by Dr. G.R.B. Skinner of the Department of Virology, The Medical School, Birmingham, England.

Summary of the test

Type-specific antigen of HSV-1 and HSV-2 were prepared by rigorous absorption of HSV-1 and HSV-2 infected cell extracts with heterotype immune sera. The sera under test were heat inactivated and tested at a dilution of 1:10 by complement fixation assay against the following five antigens:

1. Control antigen prepared with uninfected cells.
2. General virus antigen (unabsorbed) prepared from HSV-1 infected cells.
3. General virus antigen (unabsorbed) prepared from HSV-2 infected cells.
4. HSV-1 type specific antigen.
5. HSV-2 type specific antigen.

MATERIALS AND METHODS

Viruses

a) HSV-1 - The HFEM derivative if the Rockerfeller strain of HF was used.

b) HSV-2 - Strain 3345, a penile isolate from the venereal disease clinic of the General Hospital, Birmingham.

Preparation of general virus antigen

Rabbit kidney (RK13) cells were infected at 10 pfu/cell with HSV-1 or HSV-2 and incubated at 37°C for 24-36 hours. The infected cells were removed from the glass, washed with PBS and resuspended in veronal buffer (VB) at a concentration of 10^8 cells per ml and disrupted ultrasonically. The antigen thus prepared when tested by immunodiffusion against homologous immune sera gave multiple precipitin lines. The antigen was stored at -70°C.

Immunodiffusion test

This was carried out in 1% w/v ionagar in isotonic saline containing 0.1% sodium azide. Six hexagonally arranged wells surrounded a central well, each separated by 2 mm from margin to margin. The plates were incubated at room temperature in a humidified chambered for 72 hours before reading.

Preparation of control antigen

Control antigen from uninfected RK13 cells was prepared in the same manner as general virus antigen was prepared.

Preparation of type-specific antigen

1 ml of general virus antigen prepared as described above was diluted five-fold with VB and mixed with 0.25 ml of undiluted heterotypic serum. It was gently agitated at 4°C for 12 hours. The precipitate was removed by centrifuga-

tion at 3000 rpm for 15 minutes in a bench centrifuge at 4°C. This absorption procedure was repeated three times more and the final process of centrifugation carried out at 100,000 x g for one hour at 4°C. This was to remove insoluble complexes and virus particles. The antigen was stored at -70°C in 0.2 ml volumes. No decline in complement fixing activity was observed during three months' storage at this temperature.

Checking the type specific antigen by immunodiffusion

The type specific antigen gave no detectable precipitation lines on testing at different concentrations against heterotypic serum by immunodiffusion.

Preparation of antiserum to HSV-1 and HSV-2

This method was as described by Watson et al (1966).

HSV-1 and HSV-2 general virus antigen was prepared as described above. Rabbits were given three weekly intramuscular injections of 200 mg of antigen in Freund's incomplete adjuvant and thereafter a booster injection at three-monthly intervals.

Serological proceedure

A microtest was carried out in Linbro trays.

Complement: Guinea pig (Wellcome).

4 haemolytic doses were used.

Sheep red cells: The sensitized sheep red cells were used at a concentration of 5×10^7 cells per ml.

Antigen: 8 complement fixing units of antigen were used.

Sera: Heat inactivated (56°C for ½ hour) at a 1:10 dilution was used and tested in triplicate.

Antigen, serum and complement were left at 4°C overnight for fixation to occur. On the next day the indicator red cells as well as plates were brought to 37°C and the plates were further incubated for 30 minutes at 37°C, and 30 minutes at 4°C, after adding the red cells.

Controls

The controls used in the assay were:

a) control antigen
b) positive control serum for HSV-1 antibody
c) positive control serum for HSV-2 antibody
d) seronegative serum
e) complement control
f) cell control
g) antigen control

Reading of results

The results were read as indicated in Table 4.2.

Table 5.2 Classification of serotypes according to the complement fixation test.

CONTROL ANTIGEN	GENERAL VIRUS ANTIGEN HSV-1	GENERAL VIRUS ANTIGEN HSV-2	HSV-1 TYPE SPECIFIC ANTIGEN	HSV-2 TYPE SPECIFIC ANTIGEN	SERÓTYPE
	-	-	-		SN
	-	+	+	-	HSV-1 specific antibody
	-	+	-	+	HSV-2 specific antibody
	-	+	+	+	HSV-1 & HSV- specific antibody
	-	+	-	-	Seropositiv but no type specific antibody (NTSA)
	-	+	-	-	
	-	-	+	-	

SN = seronegative

METHOD 5

DETERMINATION OF LEVELS OF SERUM IgG AND IgA BY SINGLE RADIAL IMMUNODIFFUSION

METHOD FOR DETERMINING LEVELS OF SERUM IgG AND IgA BY SINGLE RADIAL IMMUNODIFFUSION

All equipment and materials used are described in Appendix G.

Method

The method described by Mancini *et al* (1965) was employed. Single radial diffusion plates (G1), reference sera (G2), and the sera to be tested were left on the bench for at least 15 minutes until they came to room temperature. The test serum samples used were fresh aliquots which had not been inactivated or frozen and thawed repeatedly. If moisture was present in the wells of the plates when the resealable plastic envelope was removed, they were allowed to stand a further 15 minutes uncovered to permit evaporation. Ten microlitre quantities of each standard reference serum (high, mid-range and low) were pipetted into three separate wells on each plate. The test sera were pipetted in 10 µl quantities into the other wells. In doing this the wells were filled from the bottom upwards. The covers of the plates were replaced and the plates allowed to stand 15 minutes before moving. The plates were placed in resealable plastic envelopes and left at room temperature on a level area for four days to allow complete diffusion. At the end of four days the diameter of the developed rings were measured to the nearest 0.1 mm, using a diameter measuring template (G4) placed over a X-ray viewer (G5). A calibration curve was constructed using the readings given by the three standard sera. The concentration in iu/ml was used as the Y-coordinate and the

ring diameter^2 as the X-coordinate. A straight line was drawn of best fit for all the points. The concentration of IgG or IgA of the test serum was read in iu/ml from the standard curve. A new standard curve was prepared for each set of plates used.

METHOD 6

ASSAY FOR CARCINO EMBRYONIC ANTIGEN (CEA)

ASSAY FOR CARCINO EMBRYONIC ANTIGEN (CEA)

Serial samples of serum collected from British patients with invasive carcinoma and single samples of serum from matched controls were assayed for levels of CEA by Dr. Frances Searle, at the Department of Oncology, Charing Cross Hospital (Fulham), Fulham Palace Road, London.

CEA was prepared by conventional perchloric acid extraction from liver metastases of colonic carcinoma. This was further fractionated by Concanavalin A chromatography. The CEA fraction released by 2% methyl glucoside (termed the 2B fraction) was labelled with ^{125}I using a modification of the chloramine-T method of Hunter and Greenwood (1962).

Rabbit antiserum was raised against this 2B fraction. This antiserum was absorbed against pooled normal human plasma and bound to enzacryl. The assay used was a solid phase direct competition assay. 50 µl of enzacryl containing bound rabbit anti-CEA serum was made to react at 37°C overnight with 100 µl of the sample, 50 µl of labelled CEA (2B fraction), and 100 µl of PBS. At the end of the overnight incubation, the reaction mixture was filtered on a rapid automated Kentek 3000 machine which directly estimated the CEA activity of the sample. The range of CEA activity recognised by the machine was 5 g/litre to 1000 g/litre. There was an inter-assay variation of 15%.

METHOD 7

DETERMINATION OF RUBELLA HAEMAGGLUTINATION INHIBITING ANTIBODY

METHOD FOR DETERMINING RUBELLA HAEMAGGLUTINATION INHIBITING ANTIBODY

All materials and equipment required for this test are described in Appendix H.

Pre-treatment of sera

1. Removal of heat labile inhibitors.

A 1:4 dilution of serum was prepared by diluting 0.2 ml of serum in 0.6 ml of haemagglutination inhibition (HAI) diluent (H3) and heat inactivated at 56°C for 30 minutes.

2. Removal of β-lipoprotein inhibitors of rubella haemagglutinin

0.01 ml of heparin (5000 units/ml) and 0.1 ml of $MnCl_2$ (1M) were added, the mixture shaken vigorously, and incubated at 4°C for 20 minutes. The precipitate was removed by centrifugation at 2000 rpm for 10 minutes.

3. Removal of excess $MnCl_2$ which causes agglutination of red cells

0.05 ml of Na_2CO_3 (0.5 M) was added, shaken vigorously and the precipitate removed by spinning at 2000 rpm for 20 minutes.

Antigen

The antigen (H2) was dissolved in HAI diluent (8-10 ml) and doubling dilutions made in duplicate using 0.025 volumes. 0.025 ml of red cells were added to each dilution, incubated at 4°C for one hour and used at a concentration of 8 units. One unit was the least amount of antigen that caused 100% agglutination of red cells.

Cells

One day old chick red cells were used which were washed in HAI diluent till the supernate was clear. A 0.25% suspension of red cells in HAI diluent was prepared.

Plates

V-shaped microtitre plates (H1) were used.

Method

1) 0.025 ml of HAI diluent was placed in all wells except the first well in each row.
2) 0.025 ml of the treated serum samples were added to the first, second and last well of each row.
3) Using standard microtitre loops which delivered 0.025 volumes, two-fold dilutions of serum were obtained starting at the second well and leaving out the last well.
4) 0.025 ml of rubella HAI antigen (8 HAU/0.025 ml) was added to all wells except the last well which served as a serum control.

5) An antigen titration was performed in each test. A low titre and high titre positive control serum and negative control serum were included in each test. A cell control too was set up.
6) The plates were incubated at RT for one hour.
7) 0.025 ml of 0.25% red cells was added to all wells.
8) Plates were incubated at $4^{o}C$ for one hour.
9) The rubella HAI titre of a serum sample was taken to be the highest dilution which provided a complete inhibition of rubella haemagglutinin.

METHOD 8

DETERMINATION OF MEASLES HAEMAGGLUTINATION INHIBITING ANTIBODY

METHOD FOR DETERMINING MEASLES HAEMAGGLUTINATION INHIBITING ANTIBODIES

These test were conducted by Dr. Mairin Clarke of the MRC National Institiute for Biological Standards and Controls.
All materials and equipment used are described in Appendix H.

Pre-treatment of sera

0.2 ml of serum was diluted in 0.2 ml of veronal buffer (VB) (H4) and adsorbed with 0.05 ml of a 50% suspension of rhesus monkey RBC at $4^{o}C$ for one hour shaking frequently. The cells were removed by centrifugation at 2000 rpm for 20 minutes on a bench centrifuge.

Antigen

Measles haemagglutinin was titrated in 3 x $\frac{1}{2}$ inch tubes. 0.7 ml of VB was added to the first of a series of 12 tubes and 0.4 ml of diluent into the rest. 0.1 ml of antigen was added to the first tube and doubling dilutions of antigen prepared.
0.2 ml of 1.0% washed monkey RBC was added to each tube.
A cell control was set up containing 0.4 ml diluent and 0.2 ml RBC.
Tubes were incubated at $37^{o}C$ for two hours.
One unit of antigen was considered to be the least amount of antigen which agglutinated 50% of red cells.
Four units were used in testing serum samples.

Plates

V-shaped microtitre plates (H1) were used.

Method

1) 0.025 ml of VB was added to all wells.

2) 0.025 ml of adsorbed serum was added to the first well and the last well in the row.

3) 0.025 ml of antigen was added to all wells except to the last which served as a serum control.

4) The plates were left at RT for one hour.

5) 0.025 ml of 1% monkey RBC was added to all wells and shaken well.

6) Plates were incubated at 37°C for two hours approximately, until the cells settled.

A positive and negative serum were included in each test. An antigen titration was done in every test and a cell control was also set up.

7) The measles HAI titre of a serum sample was taken as the highest dilution which provided a complete inhibition of measles haemagglutinin.

Chapter 6

RESULTS

RESULTS. PART I.

ANALYSIS OF AGE, SOCIAL CLASS, ETHNIC ORIGIN, AND SEX RELATED VARIABLES IN PATIENTS WITH CERVICAL ANAPLASIA, IN BRITAIN, MALAWI, SUDAN AND SRI LANKA.

PART I.

ANALYSIS OF AGE, SOCIAL CLASS, ETHNIC ORIGIN, AND SEX RELATED VARIABLES IN PATIENTS WITH CERVICAL ANAPLASIA, IN BRITAIN, MALAWI, SUDAN AND SRI LANKA.

AGE DISTRIBUTION OF PATIENTS WITH CERVICAL ANAPLASIA (dysplasia carcinoma in situ and invasive carcinoma) AND STAGE OF DISEASE OF INVASIVE CARCINOMA PATIENTS WHEN FIRST DETECTED

All three types of lesions were detected in a wide range of ages (Table 6.1). The age group showing maximum incidence among patients with dysplasia, carcinoma in situ, and invasive carcinoma in Britain was 21-30, 31-40 and 51-60 years respectively.

Incidence of cervical anaplasia among younger women in Britain.

Thirty-nine of 166 (23%) patients with cervical anaplasia were aged 30 years or younger (Table 6.2). Of these, 23 had dysplasia, 11 had carcinoma in situ, and five had invasive carcinoma, i.e. 16 (14%) of a total of 114 patients requiring immediate treatment (patients with carcinoma in situ and invasive carcinoma) were 30 years or younger.

Incidence of cervical anaplasia among post menopausal women in Britain.

Thirty-six of 70 patients (51%) with invasive car-

Table 6.1

Age distribution of patients at time of detection of pre-malignant or malignant state

Patient Group	Dysplasias Britain	Ca *in situ* Britain	Inv. Ca Britain	Inv. Ca Sri Lanka	Inv. Ca Sudan	Inv. Ca. Malawi
No. of patients	52	44	70	32	27	27
<20 yrs.	4%	-	-	-	-	-
21-30 yrs.	40%	25%	7%	6%	4%	8%
31-40 yrs.	27%	37%	14%	16%	22%	36%
41-50 yrs.	21%	27%	19%	22%	44%	28%
51-60 yrs.	4%	9%	30%	41%	15%	20%
61-70 yrs.	-	2%	21%	9%	11%	4%
>70 yrs.	4%	-	9%	6%	4%	4%
Median	29	37	53	52.5	50	43
Average	35	37.5	52.7	51	47	46.2
Range	19-79	22-68	22-80	25-74	27-75	26-80

☐ Two age groups showing maximum incidence

cinoma were aged 50-70 years and seven of the 12 (58%) patients who died as a result of recurrence were also in this age group.

Stage of disease at time of detection and maximum age of incidence in Sri Lankan, Malawian and Sudanese patients

34% of British patients with invasive carcinoma were detected in stage 1 of the disease. The proportion of patients detected in stage 1 in Sri Lanka (6%), Malawi (4%) and Sudan (none) were noticeably less (Table 6.3). However, Table 6.1 shows that in spite of presenting at later stages, the Malawian and Sudanese patients had the youngest age of maximum incidence (31-40 and 41-50 respectively). The maximum age of incidence for Sri Lankan and British patients with invasive carcinoma was 51-60 years. However, considering the age group showing the next highest incidence, 41-50 for Sri Lankan patients and 61-70 for British patients, it is possible that the Sri Lankan patients present at a younger age.

SOCIAL CLASS DISTRIBUTION OF PATIENTS WITH CERVICAL ANAPLASIA

The majority of patients from Sudan, Malawi and Sri Lanka were from the low social classes (Table 6.4).

Social class analysis of the 166 British patients showed that a major proportion of patients with dysplasia (75%), carcinoma *in situ* (73%) and invasive carcinoma (60%), were in social classes I-III. However, in comparison with the small proportion of patients with dysplasia (8%) and carcinoma *in situ* (20%) in social classes IV and V, there were more patients with invasive carcinoma (30%) in these two social classes.

Table 6.2

Analysis of British patients below the age of 30 years in relation to the total number of patients presenting with dysplasia, carcinoma in situ and invasive carcinoma

	TOTAL	NUMBER BELOW AGE OF 30 YEARS
Patients with dysplasia, carcinoma *in situ* and invasive carcinoma.	166	39 (23.4%)
Patients with carcinoma *in situ* and invasive carcinoma.	114	16 (14%)

Table 6.3

Analysis according to stage of disease at time of detection.

Invasive carcinoma patients from Britain, Sri Lanka, Malawi and Sudan

	No. of patients	Stage I	Stage II	Stage III	Stage IV
BRITAIN	64	22 (34%)	26 (41%)	13 (20%)	3 (5%)
SRI LANKA	32	2 (6%)	16 (50%)	14 (44%)	
MALAWI	27	1 (4%)	13 (48%)	4 (15%)	9 (33%)
SUDAN	27	None		Majority	Few

Table 6.4

Analysis according to social class, of patients with cervical anaplasia from Britain, Sri Lanka, Malawi and Sudan

	Total no. of patients	SOCIAL CLASS I	II	IIIA	IIIB	IV	V	not classified
DYSPLASIA Britain	52	4%	15%	23%	33%	6%	2%	17%
			75%			8%		
Ca IN SITU Britain	44	7%	7%	18%	41%	9%	11%	7%
			73%			20%		
INV. Ca Britain	70	1%	14%	16%	29%	11%	19%	10%
			60%			30%		

		SOCIAL CLASS Upper	Middle	Low
INV. Ca SRI LANKA	32		3%	97%
INV. Ca MALAWI	27		21%	79%
INV. Ca SUDAN	27			100%

ETHNIC ORIGIN OF PATIENTS WITH CERVICAL ANAPLASIA

Sri Lankan, Sudanese and Malawian patients

10% of invasive carcinoma patients from Sri Lanka were Tamil. This proportion is similar to the proportion of Tamils in the general population. The Sudanese and Malawian patients were homogeneous with regard to ethnic origin (Table 6.5).

British patients

19.6% of patients with dysplasia, 10% of patients with carcinoma *in situ*, and 10.5% of British patients with invasive carcinoma were negroid in origin (mainly West Indian) (Table 6.5). Of the 39 British patients with cervical anaplasia who were 30 years or younger, 8 (21%) were West Indian (Table 6.6). Their socio-economic distribution was similar to that of caucasians in this age group, i.e. approximately 80% of both caucasians and West Indians below the age of 30 years were in social classes I-III. In contrast with caucasians however, a higher proportion of West Indians had had a) previous venereal infection (37% v 13%), b) spontaneous or therapeutic abortion (50% v 26%) and a lesser proportion of West Indians in this age group were married.

Table 6.5

Proportions of different ethnic groups among patients with abnormal cervical cytology in Britain, Sri Lanka, Malawi and Sudan

BRITAIN			SRI LANKA	MALAWI	SUDAN
Dysplasia	Ca *in situ*	Inv. Ca			
11N (21%)	7N (16%)	7N (10.5%)	2T (10%)	32N 100%	27 Sud. 100%
41C (79%)	37C (84%)	61C (89.5%)	30S (90%)		

N - Negroid C - Caucasian T - Tamil S - Sri Lankese Sud-Sudanese

Table 6.6

Social class and sex related variable analysis of British patients below 30 years with cervical anaplasia

	TOTAL DYSPLASIA CA IN SITU INVASIVE CA		TOTAL DYSPLASIA CA IN SITU INVASIVE CA
	Caucasian	Negroid	Caucasian and Negroid
Total	31 (79%)	8 (21%)	39
Social Class I	1	1	2
II	5	2	7
IIIA	11	1	12
IIIB	9	4	13
IV	2	-	2
V	2	-	2
not known	1	1	2
Married	19 (61%)	1 (13%)	20 (51%)
Married more than once or divorced	3	-	3
Number who had become pregnant	16 (52%)	6 (75%)	22 (56%)
Number who had TOP or abortion	8 (26%)	4 (50%)	12 (31%)
P.H./O. venereal infection	4 (13%)	3 (37%)	7 (15%)

TOP - termination of pregnancy

P.H./O. - previous history of

AGE AT MARRIAGE OF PATIENTS WITH CERVICAL ANAPLASIA AND THEIR CONTROLS

A high proportion of Malawian patients and controls (47% and 45% respectively) (Table 6.7) were married before the age of 18 years. Sri Lankan patients married earlier than controls. The average age of marriage of all groups of British patients with cervical anaplasia was similar. Unfortunately, at the time British control sera were collected, the age of marriage was not determined.

Table 6.7

Analysis of age of marriage of patients with cervical anaplasia from Britain, Sri Lanka and Malawi and controls from Sri Lanka and Malawi

	CASES		CONTROLS	
	Age at marriage	Proportion married before 18 years	Age at marriage	Proportion married before 18 years
DYSPLASIAS (33) Britain	Median 21 Average 21.7 Range 17-33	$\frac{1}{33}$ (3%)	NOT KNOWN	
CA IN SITU (39) Britain	Median 20 Average 21.2 Range 17-36	$\frac{4}{39}$ (10%)	NOT KNOWN	
INV. CA (27) Britain	Median 21 Average 22 Range 17-35	$\frac{5}{61}$ (8%)	NOT KNOWN	
INV. CA (27) Sri Lanka	Median 18 Average 17.6 Range 11-22	$\frac{13}{27}$ (48%)	Median 23 Average 23.5 Range 15-35	$\frac{1}{15}$ (6.7%)
INV. CA (15) Malawi	Median 18 Average 18 Range 11-24	$\frac{8}{15}$ (47%)	Median 18 Average 18.4 Range 14-25	$\frac{5}{11}$ (45%)

RESULTS. PART II.

ON FIRST SERUM SAMPLES. IgG AND IgA ANTIBODIES TO THE MEMBRANE ANTIGENS OF HSV-2 INFECTED CELLS

PART II - FIRST SERUM SAMPLES.

IgG AND IgA ANTIBODIES TO THE MEMBRANE ANTIGENS OF HSV-2 INFECTED CELLS.

PROPORTION OF PATIENTS AND CONTROLS WITH IgG AND IgA ANTIBODIES TO MEMBRANE ANTIGENS (MA) OF HSV-2 INFECTED CELLS

A high proportion (87%-100%) of patients in all groups, as well as controls possessed IgG anti-MA antibodies (Figure 6.1).

In contrast (Figure 6.2) a significantly higher proportion of patients with dysplasia, carcinoma *in situ*, and invasive carcinoma from Britain and Sri Lanka, had IgA anti-MA when compared with controls and with patients from Britain with other genital malignancies as well as those with extra-genital non-squamous malignancies. A high proportion of British patients with invasive carcinoma who had been treated 1-12 years previously still possessed these antibodies. Similar proportions of Malawian patients and controls possessed IgA anti-MA. A high proportion of invasive carcinoma patients from Sudan for whom no controls could be obtained, also possessed these antibodies. However, the proportion of patients with non-genital squamous carcinomas who possessed IgA anti-MA was similar to that of patients with cervical anaplasia (dysplasia, carcinoma *in situ* and invasive carcinoma).

Of 174 patients with cervical anaplasia from all countries, six (3%) had no detectable IgG anti-MA, and 35 (20%) had no detectable IgA anti-MA (Table 6.8). Three Sri Lankan patients and three British patients had no detectable antibody by both the IgG and IgA assays. The corresponding number among British controls was five. All the Sri Lankan controls had antibodies by both assays.

FIGURE 6.1

IgG ANTIBODIES TO MEMBRANE ANTIGEN OF HSV-2 INFECTED CELLS IN PATIENTS WITH CERVICAL ANAPLASIA AND MATCHED CONTROLS

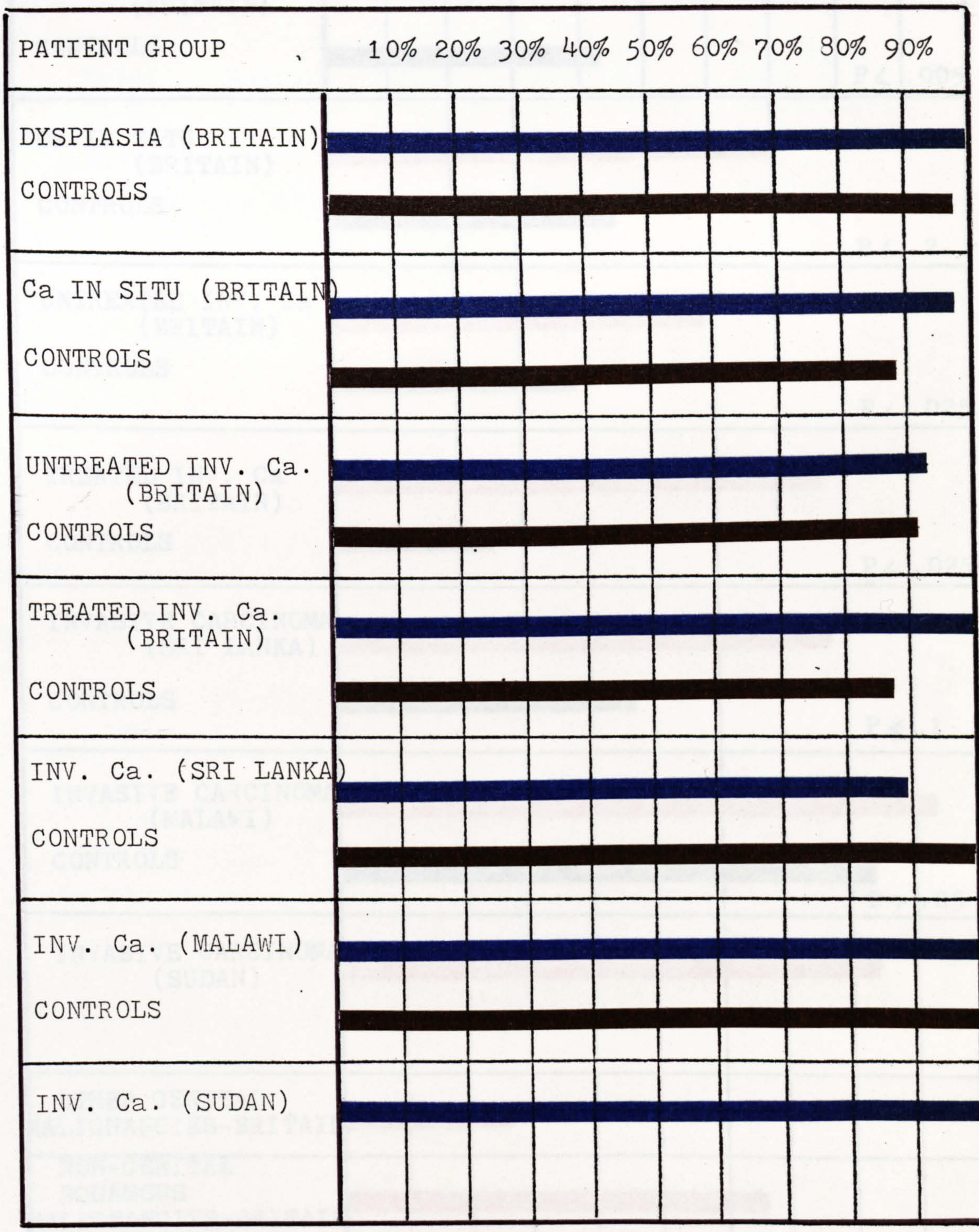


FIGURE 6.2

IgA ANTIBODIES TO MEMBRANE ANTIGENS OF HSV-2 INFECTED CELLS IN PATIENTS WITH CERVICAL ANAPLASIA, MATCHED CONTROLS AND PATIENTS WITH MALIGNANCIES OTHER THAN CERVICAL CARCINOMA

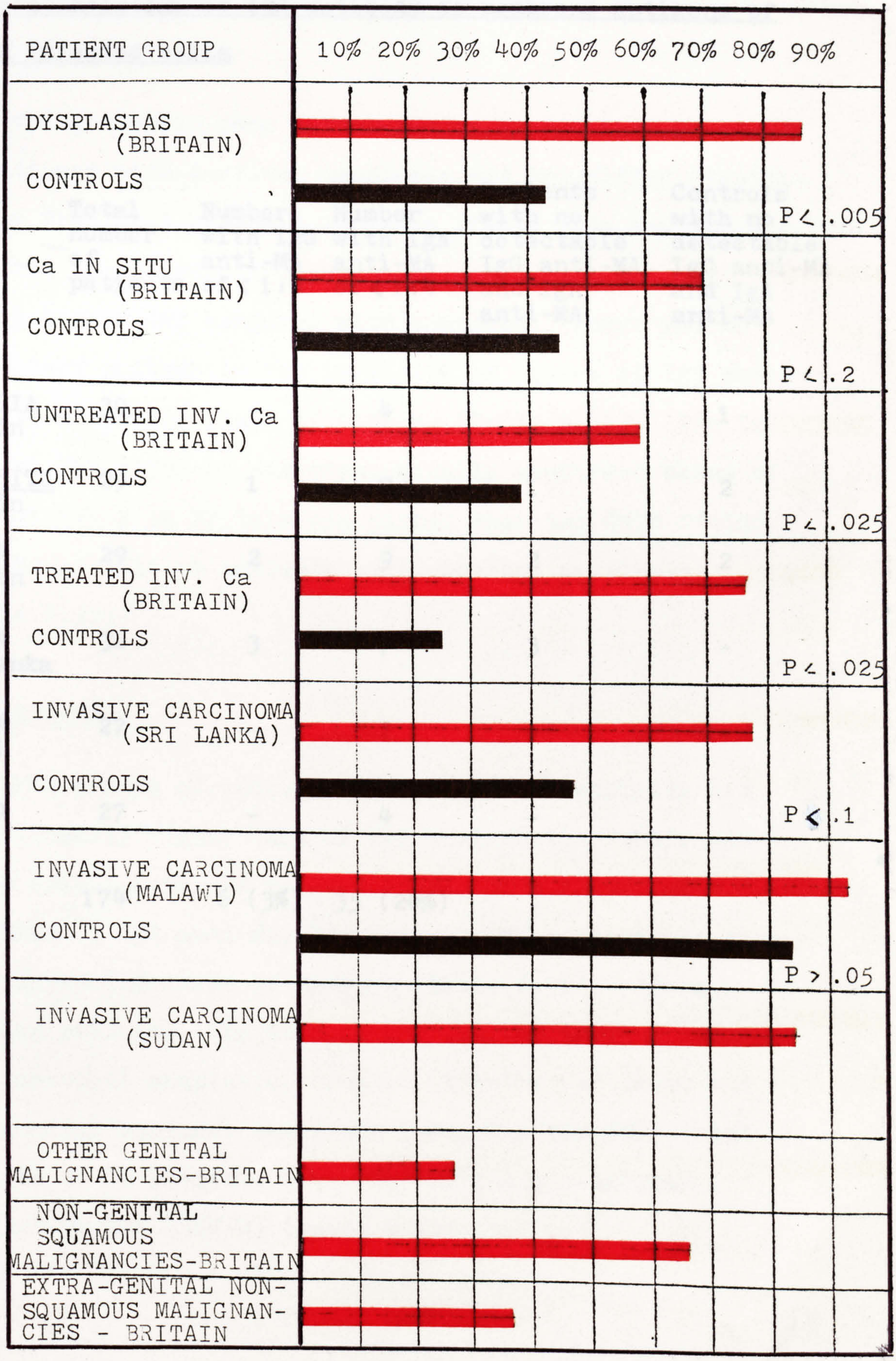


Table 5.3

Number of patients with cervical anaplasia having no detectable IgG or IgA antibody to membrane antigens of HSV-2 infected cells

	Total number of patients	Number with IgG anti-MA of < 1:8	Number with IgA anti-MA of < 1:4	Patients with no detectable IgG anti-MA and IgA anti-MA	Controls with no detectable IgG anti-MA and IgA anti-MA
DYSPLASIA Britain	30	-	4		1
CA IN SITU Britain	29	1	9	1	2
INV. CA Britain	29	2	9	2	2
INV. CA Sri Lanka	32	3	7	3	-
INV. CA Malawi	27	-	2	-	-
INV. CA Sudan	27	-	4	-	
TOTAL	174	6 (3%)	35 (20%)		

THE GEOMETRIC MEAN TITRES OF IgG and IgA ANTIBODIES TO MEMBRANE ANTIGENS (MA) OF HSV-2 INFECTED CELLS

IgG anti-MA

The geometric mean titres (GMTs) of IgG anti-MA in patients with cervical anaplasia did not differ significantly from that of their controls (Table 6.9a and Table 6.10a), with the exception of Malawian patients who had a significantly higher GMT compared with controls. There was no consistent pattern in the variation of titres of IgG anti-MA with advancing stage of disease (Table 6.11). The IgG anti-MA GMT (256) of six virologically confirmed cases of genital HSV-2 in Britain was higher than the GMTs of IgG anti-MA of British patients with cervical anaplasia (Table 6.9c).

IgA anti-MA

All groups of patients with cervical anaplasia had significantly higher GMTs of IgA anti-MA than their controls (Table 6.9b and Table 6.10b). In contrast with the GMTs of IgG anti-MA, IgA anti-MA GMT appeared to rise with advancing stage of disease (Table 6.11). Among British patients, the GMTs of IgA anti-MA in patients with cervical anaplasia, treated invasive carcinoma and non-genital squamous carcinoma resembled the GMT of IgA anti-MA of 16 patients with virologically confirmed genital herpes (HSV-2) (Table 6.9c).

Table 6.9

GMTs of IgG and IgA antibodies to membrane antigens of HSV-2 infected cells - British patients

a. IgG anti-MA

	Dysplasia	CA in situ	Invasive carcinoma
Cases	185.2 (30)	113.5 (29)	108.2 (29)
Controls	198.0 (27)	73.8 (29)	116.4 (22)
p*1	>.05	>.05	>.05

b. IgA anti-MA

	Dysplasia	CA in situ	Invasive carcinoma
Cases	8 (30)	8 (29)	9 (29)
Controls	3.8 (26)	3.5 (29)	4.2 (23)
p*1	<.005	<.01	<.05

c. IgG anti-MA and IgA anti-MA

	Genital Herpes	Other genital malignancies	Extra-genital non-squamous malignancies	Treated inv. CA	Non-gen. squamous carcinoma
IgG	256 (6)*2	79.4 (16)		106.4 (30)	
IgA	7.1 (16)*3	3.9 (33)	3.4 (37)	7.3 (30)	8.3 (18)

*1 Paired t test - 2 tailed

( ) Number of patients

*2 18 sera from 6 patients

*3 52 sera from 16 patients

Table 6.10

GMTs of IgG and IgA antibodies to membrane antigens of HSV-2 infected cells, in patients with invasive carcinoma, and matched controls from Britain, Sri Lanka, Malawi and Sudan

a. IgG anti-MA

	Britain	Sri Lanka	Malawi	Sudan
Cases	108.2 (29)	120 (32)	692 (27)	331 (27)
Controls	116.4 (22)	133 (17)	362 (18)	
p*1	>.05	>.05	>.05	

b. IgA anti-MA

	Britain	Sri Lanka	Malawi	Sudan
Cases	9 (29)	14 (32)	21.2 (27)	13 (27)
Controls	4.2 (23)	4.7 (17)	7.7 (18)	
p*1	<.05	<.02*2	<.025	

*1 Paired t-test (2 tailed)

*2 t-test for independent means. (P > .05 in a paired t-test)

Table 6.11

Analysis of GMTs of IgG anti-MA and IgA anti-MA according to the stage of disease

	IgG			IgA		
	Britain	Sri Lanka	Malawi	Britain	Sri Lanka	Malawi
Stage I	149 (9)	256 (2)	256 (1)	8(9)	11.3 (2)	8 (1)
Stage II	73.5 (10)	98.7 (16)	600.8 (13)	4.9 (10)	13,4 (16)	13.6 (1
Stage III	90.51 (8)	134.5 (14)	1024 (4)	13.45 (8)	14.5 (14)	38 (4)
Stage IV	362 (2)		812.7 (9)	64 (2)		24.5 (9

A GEOGRAPHICAL VARIATION IN THE GMTs OF ANTIBODIES TO MEMBRANE ANTIGEN

Patients and controls from Malawi possessed significantly higher IgG anti-MA GMTs than their counterparts from other countries. The IgG anti-MA GMT of patients and controls from Britain and Sri Lanka were similar, and were significantly lower than that of Sudanese patients (Table 6.12a).

A similar degree of geographical variation in GMTs was not observed when IgA anti-MA GMTs were compared (Table 6.12b). Although there was a significant difference between the values of Malawian patients and British patients, the GMTs of Malawian, Sri Lankan and Sudanese patients did not differ significantly. Similarly, Malawian controls possessed a GMT of IgA anti-MA which was significantly higher than that of British controls, but similar to those of Sri Lankan controls.

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Table 6.12

Geographical variation of GMTs of IgG anti-MA and IgA anti-MA among patients with invasive carcinoma and controls

a.

	CASES GMT of IgG anti-MA		P^{*1}	CONTROLS GMT of IgA anti-MA		P^{*1}
Britain	108.2	(29)		116.4	(22)	
Malawi	692	(27)	<.001	362	(18)	=.005
Britain	108.2	(29)		116.4	(22)	
Sri Lanka	120	(32)	>.05	133	(17)	>.05
Sri Lanka	120	(32)		133	(17)	
Malawi	692	(27)	<.001 *2	362	(18)	<.001
Sudan	331	(27)				
Malawi	692	(27)	=.001			
Sudan	331	(27)				
Britain	108.2	(29)	<.001 *2			
Sudan	331	(27)				
Sri Lanka	120	(32)	<.001 *2			

b.

	CASES GMT of IgG anti-MA		P^{*1}	CONTROLS GMT of IgA anti-MA		P^{*1}
Britain	9	(29)		4.2	(23)	
Malawi	21.2	(27)	<.025	7.7	(18)	<.05
Britain	9	(29)		4.2	(23)	
Sri Lanka	14	(32)	>.05	4.7	(17)	>.05
Sri Lanka	14	(32)		4.7	(17)	
Malawi	21.2	(27)	>.05	7.7	(18)	>.05
Sudan	13	(27)				
Malawi	21.2	(27)	>.05			
Sudan	13	(27)				
Britain	9	(29)	>.05			
Sudan	13	(27)				
Sri Lanka	14	(32)	>.05			

*1 t-test for independent means *2 Mann-Whitney - U test

RESULTS. PART III.

ON FIRST SERUM SAMPLES (cont. IgA ANTIBODIES TO VIRUS CAPSID ANTIGENS OF HSV-2 INFECTED CELLS IN PATIENTS WITH CERVICAL ANAPLASIA AND THEIR MATCHED CONTROLS.

PART III FIRST SERUM SAMPLES (cont.)

IgA ANTIBODY TO VIRUS CAPSID ANTIGEN (VCA) OF HSV-2 INFECTED CELLS IN PATIENTS WITH CERVICAL ANAPLASIA AND THEIR MATCHED CONTROLS.

In contrast with the results described in Part II on IgA anti-MA antibodies to HSV-2 infected cells, similar proportions of patients with cervical anaplasia and controls possessed IgA anti-VCA, and in similar titres, with the exception of British patients with dysplasia (Table 6.13 and Figure 6.3).

The distribution of these antibodies also did not differ significantly in patients with dysplasia who on follow up showed progressing cytology in contrast to others showing regressing cytology; nor in patients with invasive carcinoma who developed recurrence of their lesions in contrast to others who remained well (Table 6.14).

Table 6.13

Proportion of patients with cervical anaplasia possessing IgA anti-MA and IgA anti-VCA and the geometric mean titres of these antibodies

	IgA anti-MA			IgA anti-VCA		
	Proportions		GMTs	Proportions		GMTs
Dysplasias - Britain	26/30	(86.6%)	8	15/30	(50%)	19
Controls	11/26	(42%)	3.8	5/31	(16%)	12
CA *in situ* - Britain	20/29	(70%)	8	5/34	(15%)	18
Controls	13/29	(45%)	3.5	6/34	(17%)	14
Invasive CA- Britain	20/29	(69%)	9	6/30	(20%)	20
Controls	9/23	(39%)	4.2	7/28	(25%)	16
Invasive CA - Sri Lanka	25/32	(78%)	14	5/32	(15%)	18
Controls	8/17	(47%)	4.7	1/17	(5%)	
Invasive CA - Malawi	25/27	(93%)	21.2	5/25	(20%)	14
Controls	15/18	(83%)	7.7	3/15	(20%)	25

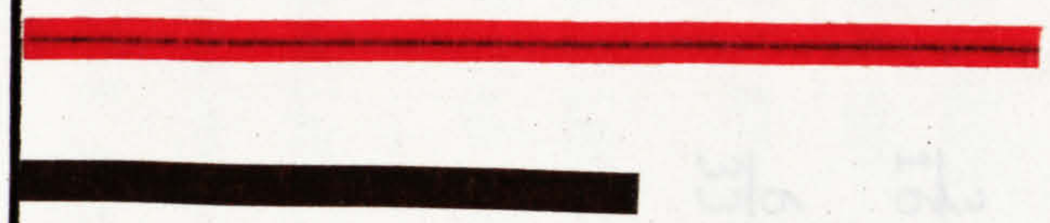
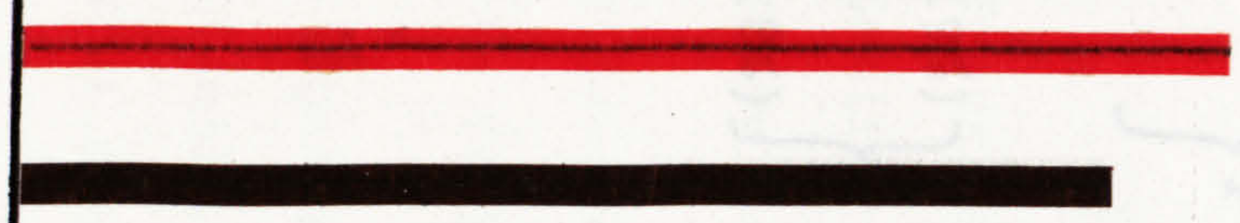
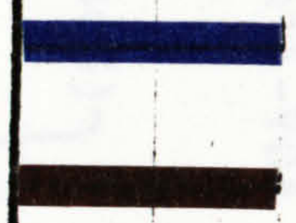
PATIENT GROUP	IgA anti-MA 10% 20% 30% 40% 50% 60% 70% 80% 90% 100%	P	IgA anti-VCA 10% 20% 30% 40% 50%	P
Dysplasias (Britain) Controls		<.00025		<.005
Ca *in situ* (Britain) Controls		>.05		>.05
Inv. Ca (Britain) Controls		<.0125		>.05
Inv. Ca (Sri Lanka) Controls		<.05		>.05
Inv. Ca (Malawi) Controls		>.05		>.05

Figure 6.3 A comparison of the prevalence of IgA anti-MA ( ) and IgA anti-VCA ( ) in patients with cervical anaplasia.

Table 6.14

Distribution of IgA anti-VCA in serum samples of patients with cervical anaplasia analysed according to their follow up histories

Group and Number	Sub-Group and Number	Proportion having IgA anti-VCA		
DYSPLASIAS (30)	Who regressed to normal cytology. (3A - N2) (15)	$\frac{6}{15}$	(40%)	P > .05
	Who had persistant 3A smears or who progressed to 3B or Pos 4. (15)	$\frac{9}{15}$	(60%)	
CA in situ OF KNOWN FOLLOW UP CYTOLOGY (29)	Normal smear after cone biopsy (22)	$\frac{5}{22}$	(23%)	P > .05
	3A smear after cone biopsy (5)	$\frac{0}{5}$		
SRI LANKAN AND BRITISH INVASIVE CARCINOMAS OF KNOWN FOLLOW UP HISTORIES (49)	Recurrence cases and terminal cases (16)	$\frac{3}{16}$	(19%)	P > .05
	Well patients (33)	$\frac{6}{33}$	(18%)	

( ) number of patients

RESULTS. PART IV

ON FIRST SERUM SAMPLES (cont'd)

HSV-1 AND HSV-2 SPECIFIC ANTIBODY.

Section 1 Prevalence of HSV-1 and HSV-2 specific antibody in all groups of patients.

Section 2 Type-specific nature of IgA anti-MA response.

Section 3 Comparison of Complement-Fixation Test and ELISA for HSV-1 and HSV-2 specific antibody.

RESULTS. PART IV. SECTION 1.

THE PREVALENCE OF HSV-2 SPECIFIC ANTIBODIES IN PATIENTS WITH CERVICAL ANAPLASIA AND IN MATCHED CONTROLS IN ALL FOUR COUNTRIES (AS DETERMINED BY ELISA)

A higher proportion of patients with cervical anaplasia from Britain, Sri Lanka and Malawi possessed HSV-2 specific antibodies when compared with controls matched for age, ethnic origin and social class (Table 6.15). This difference was significant except for the group of British patients with dysplasia ($0.1>P>0.05$). However, it is noteworthy that 27-48% of these patients did not possess HSV-2 specific antibody.

ANALYSIS OF RESULTS TO TEST THE HYPOTHESIS THAT PREVIOUS INFECTION WITH HSV-1 EXERTS A PROTECTIVE EFFECT ON THE SUBSEQUENT DEVELOPMENT OF CERVICAL ANAPLASIA

a) Prevalence of antibodies to HSV-2 alone

Among 82 patients with cervical anaplasia from Britain, Sri Lanka and Malawi and 30 controls, who possessed antibody to either HSV-1 or HSV-2 or both, there were four Sri Lankan patients and one Sri Lankan control with antibodies only to HSV-2, i.e. the proportion of patients with antibody only to HSV-2 was 4.6% in comparison with 3% among controls (Table 6.16).

b) The "inverted" case control prevalence of HSV-1 antibody alone

A higher proportion of matched controls in each country possessed only HSV-1 specific antibody without HSV-2 specific antibody when compared to patients with cervical

Table 6.15 Proportions of patients and controls having HSV-2 specific antibody as determined by ELISA

PATIENT GROUP	PROPORTION HAVING HSV-2 SPECIFIC ANTIBODY	PROPORTION AMONG CONTROLS	P (X^2 - 2 tailed)
Dysplasias (Britain)	$\frac{14}{22}$ (63%)	$\frac{11}{28}$ (39%)	>.05
Ca *in situ* (Britain)	$\frac{19}{25}$ (67.9%)	$\frac{11}{27}$ (40%)	<.05
Untreated Inv. Ca (Britain)	$\frac{13}{25}$ (52%)	$\frac{3}{24}$ (12.5%)	<.005
Treated Inv. Ca (Britain)	$\frac{19}{29}$ (65.5%)	$\frac{0}{8}$	
Inv. Ca (Sri Lanka)	$\frac{19}{29}$ (65.5%)	$\frac{4}{24}$ (16%)	<.001
Inv. Ca. (Malawi)	$\frac{19}{26}$ (73%)	$\frac{4}{13}$ (30.7%)	<.01
Inv. Ca (Sudan)	$\frac{12}{23}$ (52%)		

Table 6.16 Prevalence of antibody to HSV-2 alone among patients with cervical anaplasia from Britain, Sri Lanka and Malawi, and their matched controls (Type specific antibody by ELISA)

	NUMBER WITH ANTIBODY TO HSV-1 and HSV-2	NUMBER WITH ANTIBODY TO HSV-2 ALONE	NUMBER WITH ANTIBODY TO HSV-2 / NUMBER WITH ANTIBODY TO HSV-1& HSV-2 AND NUMBER WITH ANTIBODY TO HSV-2
Patients with cervical anaplasia in Britain, Sri Lanka and Malawi	82	4*	$\frac{4}{86}$ (4.6%)
controls from Britain, Sri Lanka and Malawi	30	1	$\frac{1}{33}$ (3%)

* Expected number 3.7

Table 6.17 Demonstrating the "inverted" case control prevalence of HSV-1 specific antibody alone (Type specific antibody by ELISA)

PATIENTS AND CONTROLS	NO. WITH ONLY ANTIBODY TO HSV-1	NO. WITH ANTIBODY TO HSV-1 AND HSV-2	NO. WITH ANTIBODY TO HSV-1 / NO. WITH ANTIBODY TO HSV-1 AND HSV-2 AND NO. WITH ANTIBODY TO HSV-1 ALONE	x 100	P X^2 (2-tailed)
Dysplasia	5	14	5/19	26%	>.05
Controls (Britain)	13	11	13/24	54%	
Ca *in situ*	5	19	5/24	20%	<.0005
Controls (Britain)	12	11	12/23	52%	
Inv. Ca	11	13	11/24	46%	<.05
Controls (Britain)	12	3	12/15	80%	
Inv. Ca	5	17	5/22	23%	>.05
Controls (Sri Lanka)	3	3	3/6	50%	
Inv. Ca	7	19	17/26	27%	<.005
Controls (Malawi)	9	2	9/11	82%	

anaplasia. This difference was significant in all groups except among British patients with dysplasia and controls and Sri Lankan patients with invasive carcinoma and controls. (Table 6.17).

THE PROPORTION OF PATIENTS WITH CERVICAL ANAPLASIA AND CONTROLS WITH NO DETECTABLE ANTIBODY TO HSV-1 OR HSV-2

Of all patients with cervical anaplasia in Britain, Sri Lanka and Malawi, 3 of 175 (1.7%) had no detectable antibody to either HSV-1 or HSV-2 by both ELISA and the IgG and IgA membrane antibody test. The proportion among controls from these three countries was 3 of 113 (2.6%) (Table 6.18).

ANALYSIS OF RESULTS TO TEST THE POSSIBILITY THAT CERVICAL ANAPLASIA DEVELOPING IN THE OLDER AGE GROUPS IS OF A DIFFERENT AETIOLOGY TO THAT DEVELOPING IN THE YOUNGER AGE GROUPS

In 102 British patients with cervical anaplasia, the prevalence of HSV-2 specific antibody was examined according to the age at which the cervical lesion was detected (Table 6.19). Sixty-six to 80% of patients in whom non-malignant (dysplasia) and malignant (Ca *in situ* and invasive carcinoma) lesions were detected below the age of 60 years possessed HSV-2 antibodies. In contrast only 37% of patients with invasive carcinoma above the age of 60 years possessed these antibodies. However, on comparing the prevalence of HSV-2 antibodies between patients and controls in the same age groups, a significantly higher proportion of the patients had HSV-2 antibodies.

Table 6.18 Proportion of patients with cervical anaplasia and controls with no detectable antibody to HSV-1 or HSV-2 by ELISA and IgG and IgA membrane antibody assays

	NUMBER	PROPORTION WITH NO ANTIBODY TO HSV-1 OR HSV-2 BY ELISA AS WELL AS IgG AND IgA MEMBRANE ANTIBODY ASSAY
All patients with cervical anaplasia from Britain, Sri Lanka and Malawi	175	$\frac{3}{175}$ x 100 = 1.7%
All controls from Britain, Sri Lanka and Malawi	113	$\frac{3}{113}$ x 100 = 2.6%

Table 6.19 Prevalence of HSV-2 specific antibody in British patients with cervical anaplasia and controls according to the age at which the lesion was detected (Type specific antibody by ELISA)

	20 - 40 yrs	40 - 60 yrs	60 - 80 yrs
Dysplasia	$\frac{12}{15}$ (80%)	$\frac{2}{3}$ (66%)	
Ca *in situ* and invasive carcinoma	$\frac{21}{30}$ (70%)	$\frac{28}{38}$ (74%)	$\frac{6}{16}$ (37%)
Controls	$\frac{18}{37}$ (49%)	$\frac{3}{18}$ (17%)	$\frac{0}{6}$ (0%)
P X^2 (2 tailed)	<.025	<.0005	<.05

There was a lower prevalence of HSV-2 specific antibody in Malawian patients over the age of 60 years in comparison with those below this age. However this same difference was not observed among Sri Lankan patients (Table 6.20).

A COHORT ANALYSIS OF THE PREVALENCE OF HSV-2 SPECICIFIC ANTIBODY (TYPE SPECIFIC ANTIBODY BY ELISA).

Figure 6.4 shows the prevalence of HSV-2 specific antibodies in cohorts of British patients with cervical anaplasia and controls. The controls in this analysis included gynaecological controls, as well as other British patients with malignancies other than cervical anaplasia.

A higher prevalence of HSV-2 specific antibodies is not seen in all cohorts of patients with cervical anaplasia when compared to cohorts of controls. The two cohorts of patients with cervical anaplasia born between 1900 and 1920 have a similar prevalence of HSV-2 antibodies as cohorts of controls born in the same period. Also surprisingly, the cohort of patients with cervical anaplasia born between 1951 and 1960 has a lower prevalence of HSV-2 antibodies than the cohort of controls born between 1951 and 1960. Among controls, a similar prevalence of HSV-2 antibodies is seen among cohorts born from 1900 to 1950 after which there is a steep rise in the prevalence from 45% among the cohort of controls born between 1941 and 1950 to 82% among the cohort of controls born between 1951 and 1960. The fact that similar prevalence of HSV-2 antibody

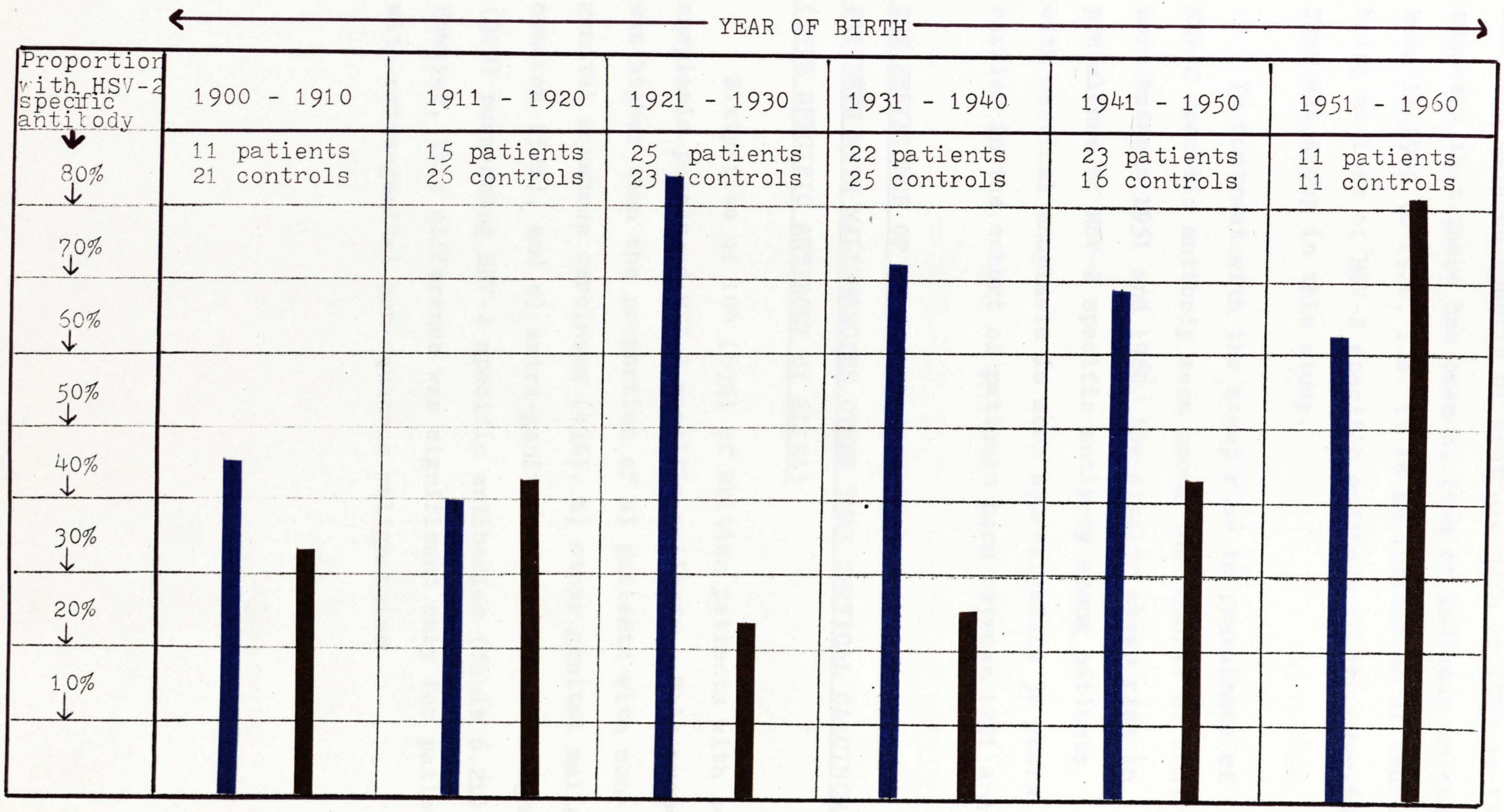


Figure 6.4 Prevalence of HSV-2 specific antibody (ELISA) in cohorts of British patients with invasive carcinoma and of British controls.

patients with invasive carcinoma

controls

was seen between cohorts of controls born in 1900 to 1950 suggests that there has been no loss of antibody in cohorts born in 1900 to 1920, i.e. it is an indication of there being no loss of HSV-2 specific antibody in the over-60 year age group in this study.

In contrast with the steep rise in prevalence of HSV-2 specific antibody seen among the cohorts of controls born between 1951 and 1960, the similar steep rise in prevalence of HSV-2 specific antibody among patients with cervical anaplasia is seen approximately 30 years earlier in the cohort of patients born between 1921 and 1930.

THE PREVALENCE OF HSV-2 SPECIFIC ANTIBODY IN BRITISH PATIENTS WITH MALIGNANCIES OTHER THAN CERVICAL CARCINOMA (TYPE SPECIFIC ANTIBODY BY ELISA)

Sixty-five of 104 (63%) of British patients with cervical anaplasia possessed HSV-2 specific antibody. This proportion was higher than the proportion of a) patients with non-genital squamous carcinoma (43%), b) other genital malignancies (52%), and c) extra-genital non-squamous malignancies (31%) possessing HSV-2 specific antibodies (Table 6.21). However, this difference was significant only for patients with extra-genital non-squamous malignancies.

Table 6.20 Prevalence of HSV-2 specific antibody (ELISA) in Malawian and Sri Lankan patients with invasive carcinoma according to age

PATIENT GROUP	<60 YEARS	>60 YEARS
Malawian patients	$\frac{18}{23}$ (78%)	$\frac{3}{5}$ (60%)
Sri Lankan patients	$\frac{15}{23}$ (65%)	$\frac{5}{6}$ (83%)

Table 6.21 Prevalence of HSV-2 specific antibody in British patients with malignancies other than cervical carcinoma

PATIENT GROUP	PROPORTION HAVING ANTIBODY TO HSV-2	PROPORTION OF BRITISH PATIENTS WITH CERVICAL ANAPLASIA WITH ANTIBODY TO HSV-2	P (2 tailed)	X^2
Non-genital squamous carcinomas	$\frac{9}{21}$ (43%)	$\frac{65}{104}$ (63%)	>.05	
Other genital malignancies	$\frac{11}{21}$ (52%)	$\frac{65}{104}$ (63%)	>.05	
Extra-genital non-squamous malignancies	$\frac{9}{29}$ (31%)	$\frac{65}{104}$ (63%)	<.005	

RESULTS. PART IV. SECTION 2.

Type-specific nature of IgA anti-MA response.

RESULTS. PART IV. SECTION 2.

TYPE SPECIFIC NATURE OF THE IgA MEMBRANE ANTIBODY RESPONSE IN HSV INFECTION

a) IgA anti-MA titres in 19 sera of known antibody type

Table 6.22 shows the IgA anti-MA and IgG anti-MA GMTs to HSV-2 infected cells, and the optical density readings (OD) by ELISA to HSV-1 specific antigen, and HSV-2 specific antigen of 19 sera. These 19 sera were tested for HSV-1 and HSV-2 type specific antibody by both ELISA and CF tests. By both assays 4 were found to contain no herpes antibody, 5 possessed only HSV-1 type specific antibody, and 11 possessed both HSV-1 and HSV-2 type specific antibody. The 11 sera containing HSV-2 antibody had a 4-fold higher IgA anti-MA GMT as well as an OD to HSV-2 specific antigen which was 19 times higher. This demonstrated the greater type specific nature of the IgA anti-MA assay because the GMT of IgG anti-MA was only 2-fold higher in the sera which possessed HSV-1 and HSV-2 specific antibody in comparison with those that possessed only HSV-1 specific antibody. Furthermore, in the 19 sera there was correlation between IgA anti-MA titres and OD readings to HSV-2 specific antigen ($P<.05$), whereas there was no similar correlation between the IgG anti-MA titres and the OD readings to HSV-2 type specific antigen. IgA anti-MA titres did not correlate with OD readings to HSV-1 specific antigen

b) IgA anti-MA in convalescent sera of HSV infected patients

Figure 6.5a shows the IgA anti-MA titres and Figure 6.5b the IgG anti-MA titres to HSV-1 and HSV-2 infected cells in convalescent sera from four patients with virologically

Table 6.22 GMT of IgG and IgA antibody to membrane antigens of HSV-2 infected cells, and mean OD value to HSV-1 specific antigen and HSV-2 specific antigen by ELISA in three groups of sera

HSV-1 AND HSV-2 TYPE SPECIFIC ANTIBODY AS DETERMINED BY CF AND ELISA	RECIPROCAL OF GMT OF IgG ANTI-MA TO HSV-2 INFECTED CELLS	RECIPROCAL OF GMT OF IgA ANTI-MA TO HSV-2 INFECTED CELLS	OD, AGAINST HSV-1 TYPE SPECIFIC ANTIGEN (ELISA)	OD, AGAINST HSV-2 TYPE SPECIFIC ANTIGEN (ELISA)
4 sera with no detectable antibodies to HSV-1 or HSV-2	13	< 4	0	0.25
5 sera with HSV-1 type specific antibody	147	3.4	17	1.2
11 sera with HSV-1 and HSV-2 specific antibody	388	16	31.5	23

CF - Complement fixation ELISA - Enzyme linked immunosorbent assay

OD - Optical density reading in ELISA

confirmed HSV-1 and five patients with virologically confirmed HSV-2 infection. Although the IgG anti-MA titres in all these 9 sera were higher to HSV-1 infected cells than to HSV-2 infected cells, the IgA anti-MA titres were in all 9 sera higher to test cells infected by the same virus type as that which infected the patient.

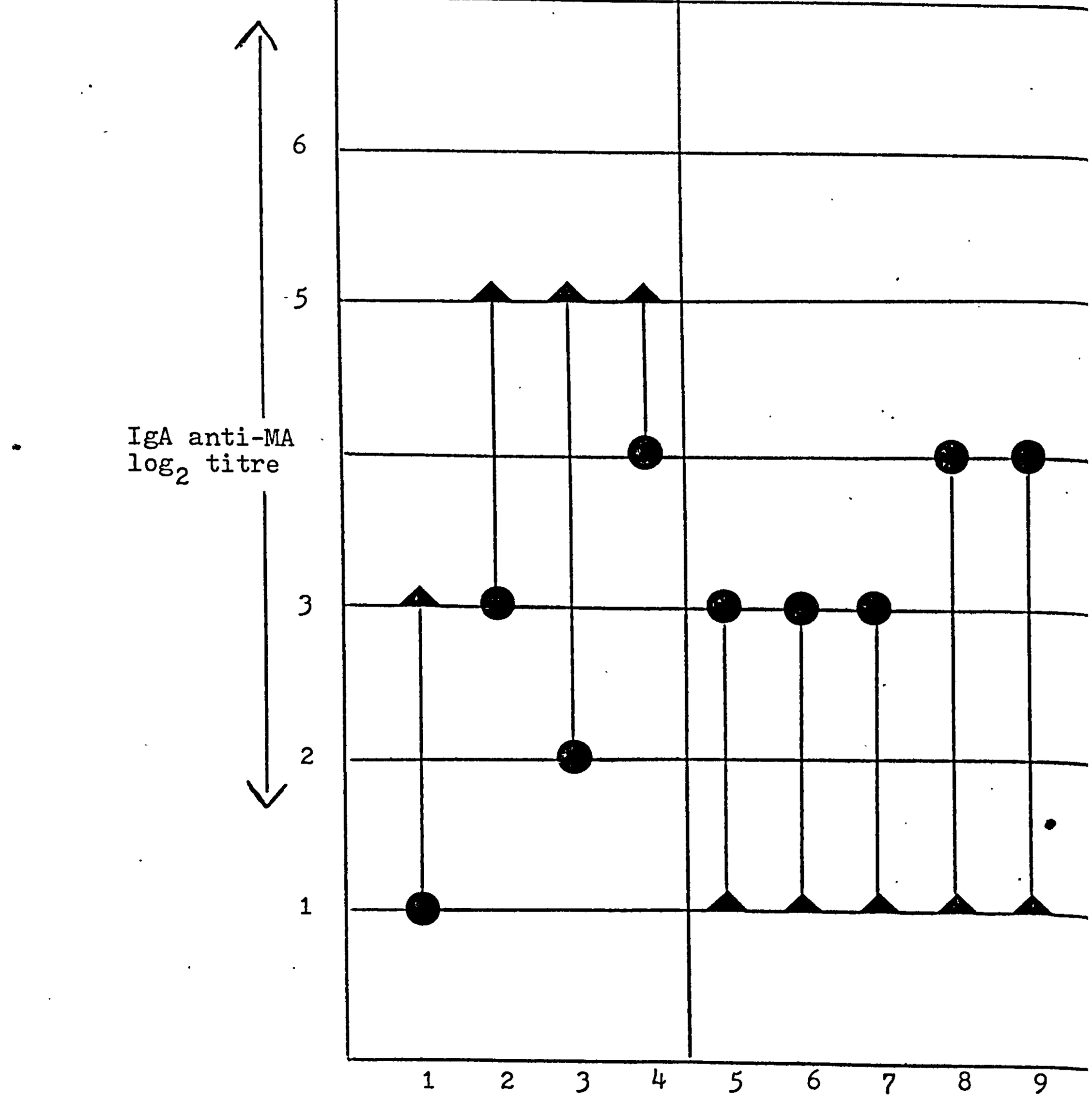


Figure 6.5a Log_2 of IgA anti-MA titres to HSV-1 infected cells (▲), and HSV-2 infected cells (●), of convalescent sera from 4 HSV-1 infected patients, and 5 HSV-2 infected patients.

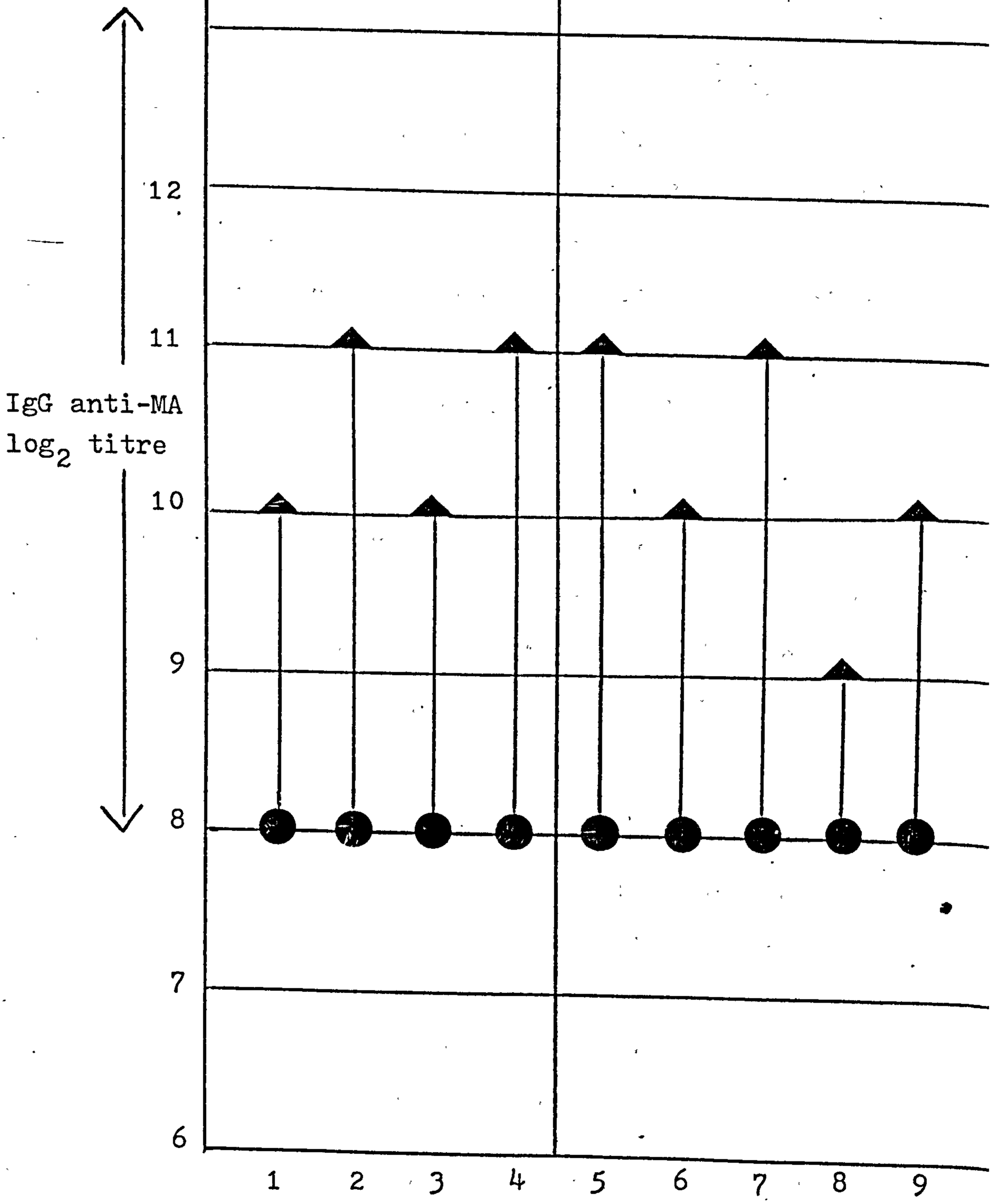


Figure 6.5b Log_2 of IgG anti-MA titres to HSV-1 infected cells (▲), and HSV-2 infected cells (●), of convalescent sera from 4 HSV-1 infected patients and 5 HSV-2 infected patients.

THE RELIABILITY OF THE IgA-MEMBRANE FLUORESCENCE ASSAY OF IgA ANTIBODY TO HSV-2 INFECTED CELLS AS A TEST OF THE PRESENCE OF HSV-2 ANTIBODY

The results in a) and b) above show that the IgA anti-MA response is more type specific than the IgG anti-MA response. Therefore, in order to determine the reliability of the IgA anti-MA assay as a test of the presence of HSV-2 specific antibody, the IgA anti-MA titres of 43 sera whose type specific antibody type was established by ELISA were examined. 21 of these sera (Figure 6.5c)possessed only HSV-1 specific antibody. 22 (Figure 6.5d) possessed both HSV-1 and HSV-2 specific antibody.

Assuming that a titre of $\geq$1:8 indicated the presence of HSV-2 specific antibody: 19 of the 21 sera which possessed only HSV-1 specific antibody had an IgA anti-MA titre of $<$1:8 and two of 21 had an IgA anti-MA titre of $>$1:8. Thus two of 21 were false positives.

Fifteen of the 22 sera which possessed HSV-1 and HSV-2 specific antibody had IgA anti-MA titres of $\geq$1:8 and seven of 22 had IgA anti-MA titres of $<$1:8. Thus the proportion of false negatives among these 22 sera was seven of 22. Therefore as a test of the presence of HSV-2 specific antibody the total false result rate was nine of 43, i.e. 20%.

The corresponding proportion of false results by CF was 34% and by ELISA 17% (?+2). However by the IgA membrane

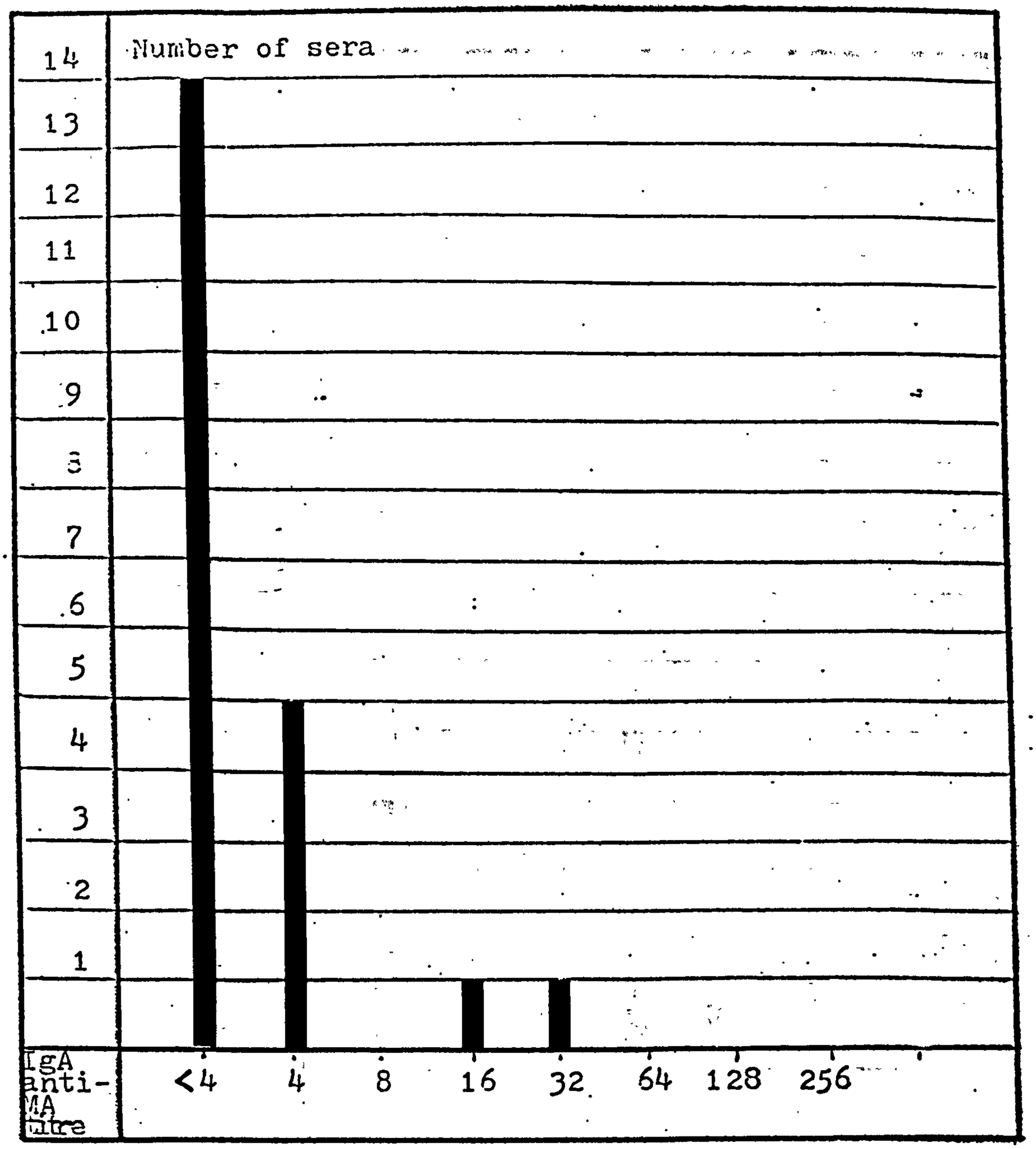


Figure 6.5c Frequency distribution of IgA anti-MA to HSV-2 infected cells in 21 British sera containing HSV-1 specific antibody by ELISA.

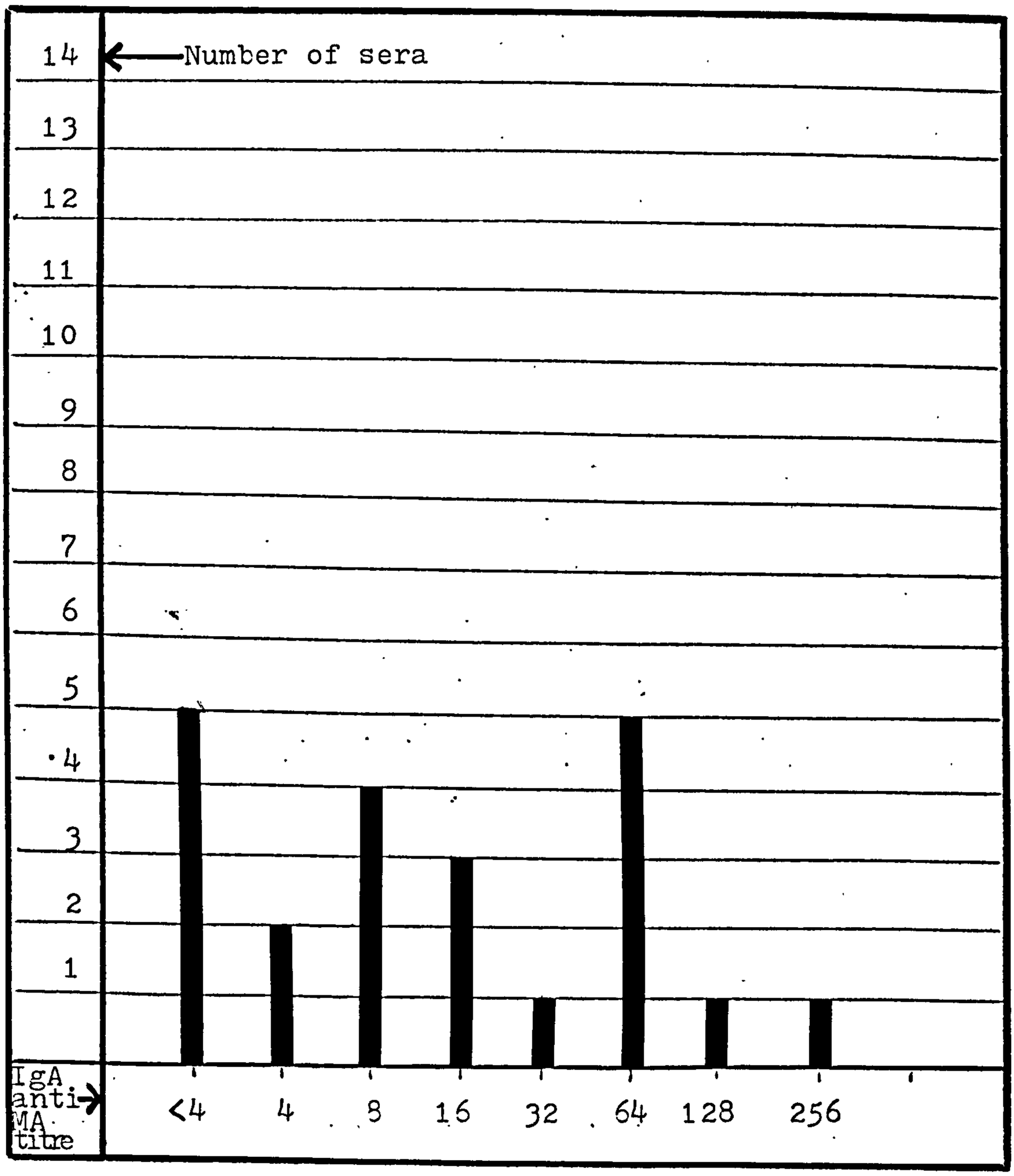


Figure 6.5d Frequency distribution of IgA anti-MA to HSV-2 infected cells in 22 British sera containing HSV-1 and HSV-2 specific antibody by ELISA.

fluorescence assay it is not possible to differentiate patients who have only HSV-2 specific antibodies from those who possess HSV-1 and HSV-2 specific antibody. Considering that in this series the proportion of patients with HSV-2 specific antibody alone was extremely small (none among British, Malawian and Sudanese patients and controls and 4 among patients and controls from Sri Lanka by ELISA), this does not appear to be such a disadvantage. Thus a British patient with an IgA anti-MA titre of $>$ 1:8 would be more likely to possess both HSV-1 specific and HSV-2 specific antibodies.

Among Sri Lankan patients and controls too, an IgA anti-MA titre of $\geq$ 1:8 differentiated patients who possessed HSV-2 specific antibody (by ELISA) from patients who did not, with a false result rate of 25%.

Among Malawian patients the corresponding differentiating titre was 1:16 with a false result rate of 17%.

A significantly higher proportion of Malawian patients with invasive carcinoma had IgA anti-MA titres of $\geq$1:16 in contrast with controls, i.e. 18 of 27 (66%) Malawian patients with invasive carcinoma possessed IgA anti-MA titres of $\geq$1:16 in contrast with 7 of 18 (38%) of controls ($P<.05$). This contrasts with the picture in Fig. 6.2 where a titre of 1:4 did not differentiate Malawian patients from controls.

THE PREVALENCE OF IgA ANTI-MA AT A TITRE OF $\geq$1:8 IN BRITISH PATIENTS WITH CERVICAL ANAPLASIA, CONTROLS, AND BRITISH PATIENTS WITH MALIGNANCIES OTHER THAN CERVICAL CARCINOMA

Employing the IgA anti-MA titre of 1:8 which was

established as explained above, it was found that a significantly higher proportion of British patients with cervical anaplasia had IgA anti-MA titres of $\geq$1:8 compared with matched controls and with patients having other genital malignancies and extra-genital non-squamous malignancies (Table 6.23). However a higher proportion of patients with non-genital squamous carcinoma possessed IgA anti-MA titres of $\geq$1:8 compared with patients with cervical anaplasia. This difference was not significant.

Table 6.23 The prevalence of IgA anti-MA at a titre of >1 :8 in British patients with cervical anaplasia, in controls, and in British patients with malignancies other than cervical carcinoma

GROUPS BEING COMPARED	PROPORTION WITH IgA ANTI-MA >1 :8	P
Cervical anaplasia	47/88 (53%)	<.01
Controls	24/78 (30%)	
Non-genital squamous carcinomas	16/24 (66%)	>.05
Cervical anaplasia	47/88 (53%)	
Other genital malignancies	9/33 (27%)	<.025
Cervical anaplasia	47/88 (53%)	
Extra-genital non-squamous malignancies	8/37 (21%)	<.005
Cervical anaplasia	47/88 (53%)	

RESULTS. PART IV. SECTION 3.

Comparison of CF test and ELISA for HSV-1 and HSV-2 type specific antibody.

RESULTS. PART IV. SECTION 3.

COMPARISON ON 58 SERA BY THE CF TEST AND ELISA, AS METHODS OF DETECTING PREVIOUS INFECTION BY HSV-1 AND HSV-2

In 20 (37%) sera (Box A. M and Y, Table 6.24) comparable results were obtained by the CF test and ELISA. However of the 4 sera in box Y which were classified as being seronegative, i.e. as having no type common or type specific antibodies by both assays, 3 contained IgG anti-MA and were therefore false seronegatives by both CF and ELISA. The 38 sera in which discordant results were obtained were reassessed in the light of IgG and IgA membrane antibody results, and the optical density (OD) results by ELISA to HSV-1 and HSV-2 type specific antigen. They were then reclassified according to the estimated HSV-1 and HSV-2 specific antibody. The aim of this exercise was:

a) to estimate the approximate false negative result rate by CF and ELISA, and

b) to estimate the distribution of HSV-1 and HSV-2 specific antibody in British patients with cervical anaplasia and controls on the basis of the combined results.

THE 38 SERA ON WHICH DISCORDANT RESULTS WERE OBTAINED BY THE CF TEST AND ELISA

HSV-1 specific antibody by CF and HSV-1 and HSV-2 specific antibody by ELISA (Box C, Table 6.24)

Table 6.24 Direct comparison of results on 58 sera by CF and ELISA

BY ELISA →

BY CF ↓	HSV-1 sp. antibody					HSV-2 sp. antibody					HSV-1 & HSV-2 sp. antibody					No type sp. antibody					No detectable type common or type sp. antibody				
	No.	GMT of IgG anti-MA	GMT of IgA anti-MA	OD-1	OD-2	No.	GMT of IgG anti-MA	GMT of IgA anti-MA	OD-1	OD-2	No.	GMT of IgG anti-MA	GMT of IgA anti-MA	OD-1	OD-2	No.	GMT of IgG anti-MA	GMT of IgA anti-MA	OD-1	OD-2	No.	GMT of IgG anti-MA	GMT of IgA anti-MA	OD-1	OD-2
HSV-1 sp antibody	A 5	147	3.3	17	1.2	B					C 7	210	4.4	37	10	D					E 2				
HSV-2 sp. antibody	F					G					H 3	512	20	41	44	I					J				
HSV-1 & HSV-2 sp. antibody	K 5	97	8	27	0	L					M 10	388	16	31.5	23	N					O				
NTSA	P 10	157	2	19	0.6	Q					R					S					T 1	16	<4	1	0
SN	U 5	111	5	19	1.25	V					W 3	203	8	42	19	X					Y 4	13	2	0	.25

3 false seronegatives by CF and ELISA

NTSA - No type specific antibody.

SN - No detectable, type common or type specific antibody.

Patient No. 1 and 2 (Table 6.25) had no detectable IgA anti-MA, and also had very low OD values to HSV-2 type specific antigen. It is possible therefore that they were false positives for HSV-2 specific antibody by ELISA. They have been considered as possessing only HSV-1 specific antibody. Numbers 3 to 7 have been considered to possess both HSV-1 and HSV-2 specific antibody, as demonstrated by ELISA and may therefore be false negatives for HSV-2 specific antibody by the CF test.

HSV-1 specific antibody by CF, seronegative by ELISA (Box E, Table 6.24)

Of the two patients in this category (Patient No. 8 and 9, Table 6.25), patient 8 had no detectable antibodies by the IgG membrane antibody assay. This was considered a false negative by the IgG membrane antibody assay and ELISA. Patient No. 9 had an IgG anti-MA titre of 1/128 and was considered to be a false negative by ELISA.

HSV-2 specific antibody by CF, HSV-1 and HSV-2 specific antibody by ELISA (Box H, Table 6.24)

In these three patients (No. 10, 11 and 12, Table 6.25), the OD with HSV-1 type specific antigen by ELISA were all reasonably high. Therefore, results by ELISA have been considered as correct, i.e. these were three false negatives for HSV-1 specific antibody by complement fixation.

HSV-1 and HSV-2 specific antibody by CF and HSV-1 specific antibody by ELISA (Box K, Table 6.24)

Of these five patients, two (No. 15 and no. 17, Table 6.25)

did not have detectable levels of IgA anti-MA. However, among the 11 patients (Box M, Table 6.24) in whom HSV-1 specific and HSV-2 specific antibody were detected by both CF and ELISA there was one who did not have detectable levels of IgA anti-MA antibody.

Therefore these five sera have been considered to be five false negatives for HSV-2 specific antibody by ELISA.

<u>No type specific antibody by CF, but HSV-1 specific antibody by ELISA (Box P, Table 6.24)</u>

These 10 patients (No. 18 to 27, Table 6.25) had a similar GMT of IgG anti-MA and mean OD to HSV-1 type specific antigen and mean OD to HSV-2 type specific antigen as the patients in Box A, Table 6.24, who had HSV-1 specific antibody by both CF and ELISA. Therefore these 10 patients have been considered as false negatives for HSV-1 specific antibody by CF.

<u>No type specific antibody by CF, but HSV-1 and HSV-2 specific antibody by ELISA (Box R, Table 6.24)</u>

These two sera (No. 28 and 29, Table 6.25) have been considered as possessing HSV-1 and HSV-2 specific antibody although serum no. 29 was a doubtful positive for HSV-2 specific antibody as there was no detectable IgA anti-MA, and the OD to HSV-2 type specific antigen was low.

<u>No type specific antibody by CF, and seronegative by ELISA (Box T, Table 6.24)</u>

This patient (No. 30, Table 6.25) had low levels of

Table 6.25 Estimation of the correct HSV antibody status of 39 sera on which discordant results were obtained by ELISA and CF, with the use of the IgG and IgA membrane antibody results

PATIENT CATEGORY IN RELATION TO TABLE	NO.	RECIPROCAL OF IgG ANTI-MA	RECIPROCAL OF IgA ANTI-MA	OD TO HSV-1 TYPE SPECIFIC ANTIGEN	OD TO HSV-2 TYPE SPECIFIC ANTIGEN	CORRECT HSV ANTIBODY STATUS OF PATIENT	BY CF ASSAY (IgG-MA ASSAY)	BY ELISA ASSAY
HSV-1 specific antibody by CF, HSV-1 and HSV-2 specific antibody by ELISA (Box C, Table 6.24)	1	64	< 4	4	4	HSV-1 specific antibody only		2 false positives
	2	256	< 4	34	6			
	3	512	4	22	15			
	4	128	4	41	10	HSV-1 and HSV-2 specific antibody	5 false negatives for HSV-2 specific antibody	
	5	256	4	26	15			
	6	128	64	27	10			
	7	512	< 4	109	12			
HSV-1 specific antibody by CF, SN by ELISA (Box E, Table 6.24)	8	< 8	< 4	0	0	HSV-1 specific antibody	(False -ve by IgG-MA assay)	False sero-negative
	9	128	< 4	0	0	HSV-1 specific antibody		False sero-negative
HSV-2 specific by CF and HSV-1 and and HSV-2 specific antibody by ELISA (Box H, Table 6.24)	10	1024	< 4	72	85	HSV-1 specific and HSV-2 specific antibody	3 false -ve for HSV-1 specific antibody by CF	
	11	128	256	16	25			
	12	1024	16	36	24			
HSV-1 and HSV-2 specific antibody by CF and HSV-1 specific antibody by ELISA (Box K, Table 6.24)	13	256	64	60	0	HSV-1 specific and HSV-2 specific antibody		5 false negative for HSV-2 type sp. antibody
	14	128	16	9	0			
	15	64	4	5	0			
	16	128	8	26	0			
	17	32	4	33	0			

PATIENT CATEGORY IN RELATION TO TABLE	NO.	RECIPROCAL OF IgG ANTI-MA	RECIPROCAL OF IgA ANTI-MA	OD TO HSV-1 TYPE SPECIFIC ANTIGEN	OD TO HSV-2 TYPE SPECIFIC ANTIGEN	CORRECT HSV ANTIBODY STATUS OF PATIENT	BY CF ASSAY (IgG-MA ASSAY)	BY ELISA ASSAY
NTSA by CF and HSV-1 specific antibody by ELISA (Box P, Table 6.24)	18	128	4	10	1			
	19	128	< 4	11	3			
	20	256	< 4	19	1			
	21	64	< 4	17	0	HSV-1 specific antibody	10 false negatives for HSV-1 specific antibody	
	22	128	< 4	20	0			
	23	256	4	43	1			
	24	128	< 4	10	0			
	25	128	< 4	5	0			
	26	256	< 4	38	0			
	27	256	< 4	19	0			
Reciprocal of GMT of membrane antibody and mean OD value	18-27	157.5	2.46	19.2	0.6			
NTSA by CF, HSV-1 and HSV-2 specific antibody by ELISA (Box R, Table 6.24)	28	32	4	23	25	HSV-1 and HSV-2 specific antibody	False negatives for HSV-1 and HSV-2 specific antibody	
	29	512	< 4	25	8			
NTSA by CF, SN by ELISA (Box T, Table 6.24)	30	16	< 4	1	0	HSV seropositive but no detectable type specific antibody		False seronegative by ELISA

PATIENT CATEGORY IN RELATION TO TABLE	NO.	RECIPROCAL OF IgG ANTI-MA	RECIPROCAL OF IgA ANTI-MA	OD TO HSV-1 TYPE SPECIFIC ANTIBODY	OD TO HSV-2 TYPE SPECIFIC ANTIBODY	CORRECT HSV ANTIBODY STATUS OF PATIENT	BY CF ASSAY (IgG-MA ASSAY)	BY ELISA ASSAY
SN by CF and HSV-1 type specific antibody by ELISA (Box U, Table 6.24)	31	64	32	23	2	HSV-1 specific antibody	5 false sero-negatives	
	32	128	16	36	0			
	33	64	<4	2	0			
	34	256	<4	26	2			
	35	128	<4	8	0			
SN by CF, HSV-1 and HSV-2 specific antibody by ELISA (Box W, Table 6.24)	36	64	<4	5	15	HSV-1 specific and HSV-2 specific antibody	3 false sero-negatives	
	37	128	23	23	4			
	38	1024	8	100	38			

IgG anti-MA antibody. This serum was therefore considered a false seronegative by ELISA.

Seronegative by CF, HSV-1 specific antibody by ELISA (Box U, Table 6.24)

All these five patients (No's 31-35, Table 6.25) had IgG anti-MA antibodies. They were therefore considered as false seronegatives by CF.

Seronegative by CF, HSV-1 and HSV-2 specific antibody by ELISA (Box W, Table 6.24)

All three patients in this category (No's 26-29, Table 6.25) had IgG anti-MA antibody. They were therefore considered false seronegatives by CF.

EFFICIENCY OF THE THREE ASSAYS (CF, ELISA, AND MEMBRANE ANTIBODY) IN DETECTING HSV-1 AND HSV-2 TYPE COMMON AND TYPE SPECIFIC ANTIBODY

Table 6.26A shows the final classification of the 58 sera compared, according to the presence or absence of HSV-1 and HSV-2 specific antibody following corrections made as explained in Table 6.25. Table 6.26B is an estimate of false negative rates per 100 sera by each assay.

1. As a method of determining antibody to the antigen common to HSV-1 and HSV-2

The IgG membrane antibody was the most sensitive (false negative rate of 1.7%). The CF test and the ELISA gave false negative rates of 19% and 10% respectively (Table 6.26B).

Table 6.26

A. Final classification of the 58 sera compared, according to HSV-1 and HSV-2 specific antibody (based on corrections made in Table 6.25).

No. of sera with HSV-1 specific antibody - 24

No. of sera with HSV-1 and HSV-2 specific antibody - 29

Total no. of sera with HSV-1 specific antibody - 53

No. of sera with no type specific antibody - 4

No. of seronegative sera - 1

B. Assessment of "false" results* by CF and ELISA assay (based on corrections made in Table 6.25)

	FALSE SERONEGATIVES (HSV-1 AND HSV-2) PER 100 SERA	FALSE NEGATIVE RATE FOR HSV-1 TYPE SPECIFIC ANTIBODY PER 100 SERA	FALSE NEGATIVE RATE FOR HSV-2 TYPE SPECIFIC ANTIBODY PER 100 SERA
CF Assay	11/58 (19%)	23/53 (43%)	10/29 (34%)
ELISA Assay	6/58 (10%)	2/53 (3.7%)	5/29 (17%) +?2/58 (3%) (false positive)
IgG membrane antibody assay	1/58 (1.7%)	—	-

* A seronegative serum by one assay was classified as false seronegative as well as false negative for HSV-1 specific antibody, if by the results of the other assay as well as the membrane antibody assays it was estimated that the serum contained HSV-1 specific antibody.

2. <u>As a method of estimating HSV-1 specific antibody</u>. ELISA was the most sensitive (false negative rate of 3.7%). The CF test was much less sensitive (false negative rate of 43%).

3. <u>As a method of estimating HSV-2 specific antibody</u> ELISA was more sensitive (17% false negative and ? 3% false positive). The CF test had a false negative rate of 34%.

<u>A comparison of the distribution of HSV-1 and HSV-2 specific antibody in 36 British patients with cervical anaplasia and 22 controls by A) the complement fixation test, B) ELISA, and C) a combined assessment based on results by CF, ELISA and IgG and IgA membrane antibody assays</u>

A significantly higher proportion of patients with cervical anaplasia possessed HSV-2 specific antibody compared with controls, by both the CF test (Column A, Table 6.27) and ELISA (Column B, Table 6.27). However, when the results by both assays and the IgG and IgA membrane antibody results on these sera were made use of to make a more accurate assessment of the herpes antibody type in each serum as explained above, the following observations were made:

A) The proportion of seronegatives among patients fell to 1/36 (3%) and among controls to none.

B) Fewer patients were classified as not possessing type specific antibody in comparison with the proportion obtained by the CF test (3% and 14% respectively).

C) There were no patients with only HSV-2 specific antibody.

D) A higher proportion (72%) of patients with cervical anaplasia had HSV-2 specific antibody in contrast to 50% by CF and 64% by ELISA.

Table 6.27 A comparison of the distribution of HSV-1 and HSV-2 specific antibody in 36 British patients with cervical anaplasia and 22 controls by A, the complement fixation assay, B, ELISA, and C, a combined assessment based on results by CF, ELISA and IgG and IgA membrane antibody assays

	A BY THE COMPLEMENT FIXATION ASSAY		B BY THE ELISA		C BY THE COMBINED RESULTS OF CF, ELISA, AND IgG AND IgA MEMBRANE ANTIBODY ASSAY	
	PATIENTS 36	CONTROLS 22	PATIENTS 36	CONTROLS 22	PATIENTS 36	CONTROLS 22
Seronegative, i.e. no antibody to type common or type-specific antigens of HSV-1 and HSV-2	5 (14%)	7 (32%)	2 (6%)	5 (23%)	1 (3%)	-
No detectable antibody to type-specific antigens of HSV-1 and HSV-2	5 (14%)	8 (36%)	-	-	1 (3%)	3 (14%)
Antibody to HSV-1 type specific antigen	8 (22%)	6 (27%)	-	-	8 (22%)	16 (72%)
Antibody to HSV-2 type specific antigen	3 (8%)	-	-	-	-	-
Antibody to HSV-1 and HSV-2 type specific antigen	15 (42%)	1 (5%)	23 (64%)	3 (14%)	26 (72%)	3 (14%)
Total number with antibody to HSV-2 stype specific antigen	$\frac{18}{36}$ (50%)	$\frac{1}{22}$ (5%)	$\frac{23}{36}$ (64%)	$\frac{3}{22}$ (14%)	$\frac{26}{36}$ (72%)	$\frac{3}{22}$ (14%)
P X^2 (2 tailed)	<.001		<.0005		<.0005	

RESULTS. PART V.

ON FIRST SERUM SAMPLES (cont'd)

LEVELS OF SERUM IgG AND IgA IN PATIENTS WITH CERVICAL CARCINOMA, CONTROLS, AND PATIENTS WITH OTHER GENITAL MALIGNANCIES

PART V

RESULTS ON FIRST SERUM SAMPLES (cont'd)

GEOMETRIC MEAN LEVELS (GMLs) OF TOTAL IgG AND IgA IN FIRST SERUM SAMPLES OF PATIENTS WITH INVASIVE CARCINOMA AND CONTROLS IN BRITAIN, SRI LANKA, MALAWI AND SUDAN; AND OF TOTAL IgA IN BRITISH PATIENTS WITH OTHER GENITAL MALIGNANCIES.

Geographical variation in levels of total IgG and total IgA.

The levels of total IgG of patients from Sri Lanka, Malawi and Sudan and controls from Sri Lanka and Malawi were similar and significantly higher than levels in patients and controls in Britain (Table 6.28).

Although within each country patients had higher IgA levels than their matched controls (Table 6.30b), these levels did not differ significantly with those of their counterparts in the other countries (Table 6.29). These findings in the British, Malawian and Sri Lankan sera were confirmed by a multi-way analysis of variance (Appendix J, 3&4) which controlled for the separate influence on the levels of total IgG and IgA by age and stage of disease.

Comparison of GMLs of total IgG and total IgA between patients with invasive carcinoma and controls within the same country.

Patients with invasive carcinoma from Britain, Sri Lanka and Malawi had GMLs of IgG which were similar to those of their controls (Table 6.30a). In contrast British and Sri Lankan patients had significantly higher GMLs of IgA when compared with controls (Table 6.30b). Malawian

Table 6.28 Statistical comparison of IgG levels among patients from Sudan, Sri Lanka, Malawi and Britain and controls from Sri Lanka, Malawi and Britain

COUNTRY	GML OF IgG IN PATIENTS	P*	GML OF IgG IN CONTROLS	P*
Britain Sri Lanka	140.7 247	< .001	131.8 241	< .001
Britain Malawi	140.7 256	< .001	131.8 277	< .001
Sri Lanka Malawi	247 256	> .05	241 277	NS
Britain Sudan	140.7 217.6	< .001		
Sudan Malawi	217.6 256	> .05		
Sudan Sri Lanka	217.6 247	> .05		

* t-test (2 tailed) for Independent Means

Table 6.29 Statistical comparison of IgA levels among patients from Britain, Sri Lanka, Malawi and Sudan, and controls from Britain, Sri Lanka and Malawi

COUNTRY	GML OF TOTAL IgA IN PATIENTS	P*	GML OF TOTAL IgA IN CONTROLS	P*
Britain	101.7	>.05	83.2	>.05
Sri Lanka	116.9		88.9	
Britain	101.7	>.05	83.2	>.05
Malawi	101.1		86.7	
Sri Lanka	116.9	>.05	88.9	>.05
Malawi	101.1		86.7	
Sudan	157.14	>.05		
Britain	101.7			
Sudan	157.14	>.05		
Malawi	101.18			
Sudan	157.14	>.05		
Sri Lanka	116.9			

Table 6.30 Geometric mean levels of total immunoglobulin (IgG and IgA) in first serum samples of invasive carcinoma patients in Britain, Sri Lanka and Malawi, and matched controls.

a) GEOMETRIC MEAN LEVELS OF TOTAL IgG

COUNTRY	BRITAIN	SRI LANKA	MALAWI	SUDAN
Cases	140.7 (28)	247 (31)	256 (25)	217.6
Controls	131.8 (27)	241 (17)	277 (16)	
Probability*1	P>.05	P>.05	P>.05	

b) GEOMETRIC MEAN LEVELS OF TOTAL IgA

COUNTRY	BRITAIN (untreated cases)	SRI LANKA	MALAWI	SUDAN	BRITAIN (treated Inv. Ca)*2
Cases	101.7 (29)	116.9 (32)	101.18 (25)	157.14 (23)	68.26 (15)
Controls	83.2 (27)	88.9 (77)	86.7 (18)		69.28 (15)
Probability	P<.01	P<.01	P>.05		

*1 Paired t-test (2 tailed)

*2 Serum samples collected 1-12 years after treatment

( ) No. of patients

patients had higher GMLs of IgA than their controls, but this difference was not significant. The GML of IgA in the treated group of British patients with cervical carcinoma was similar to that of controls (Table 6.30b).

Analysis of GMLs of IgG and IgA according to stage of disease

GMLs of both IgG and IgA showed a tendency to increase with progress of the disease.(Table 6.31 and 6.32).

In a multi-way analysis of variance which controlled for the separate effects of race and age, the increase of total IgG with progress of disease was not significant, but the increase of total IgA with progress of disease was ($P < .01$) - see Appendix J, 3 and 4.

The inverse relationship of total IgA and IgA anti-MA in invasive carcinoma patients in Britain, Malawi and Sri Lanka.

By the same multi-way analysis of variance which controlled for the effects of race, age and stage of disease, IgA anti-MA titres were inversely related to total IgA. When total IgA was doubled, IgA anti-MA was multiplied by a factor of 0.6461 (Appendix J5).

Variations in levels of total IgG and total IgA with increasing age.

The total IgG and total IgA results of 27 controls showed a tendency for IgA levels to increase with age, but after the age of 60 years, IgG levels did not show a consistent change with increasing age (Table 6.33). In con-

trast by computer analysis, levels of total IgA in patients with invasive carcinoma in Britain, Sri Lanka and Malawi decreased with age, but this effect was not significant at the .05 level (P=1).

Comparison of GML of IgA in British patients with invasive carcinoma and British patients with other malignancies.

The levels of total IgA in the pre-treatment serum samples of British patients with squamous carcinoma of the cervix was shown to be significantly higher than those of matched controls (Table 6.30b). In contrast seven patients with adenocarcinoma of the cervix, 10 patients with endometrial cancer and five patients with malignancies of the ovary had levels similar to that of controls matched for age, sex and social class (Table 6.34). Although there were only three British patients with non-cervical squamous genital carcinoma (vulval squamous carcinoma), it is of interest that these patients had levels of total IgA which were similar to those in pre-treatment sera of British patients with squamous cervical carcinoma. Matched controls for these three patients with vulval carcinoma had levels of total IgA which were similar to those of controls for the patients with squamous carcinoma of the cervix.

Table 6.31 Variation in levels of IgA with progressing stage of disease

	BRITAIN	SRI LANKA	MALAWI
Stage I	82.44 (9)	109.9 (2)	65 (1)
Stage II	101.58 (10)	109.6 (16)	93.8 (13)
Stage III	115.6 (8)	127.8 (14)	72.12 (3)
Stage IV	146.1 (2)	-	138. (8)

Table 6.32 Variation in levels of IgG with progressing stage of disease

	BRITAIN	SRI LANKA	MALAWI
Stage I	126.1 (9)	120 (1)	158 (1)
Stage II	138.7 (10)	258.6 (16)	256.2 (13)
Stage III	149.1 (8)	273.8 (14)	246.0 (3)
Stage IV	176.2 (2)	-	321.7 (8)

Table 6.33 Analysis according to age, of the levels of IgG and IgA in 27 British controls

AGE GROUP	IgG iu/ml	IgA iu/ml
21 - 30 (3)	171	78
31 - 40 (3)	162	78
41 - 50 (3)	142	76
51 - 60 (7)	128	69.4
61 - 70 (6)	100	82.4
70 (5)	140	125.01

Table 6.34 Geometric mean levels of IgA in pre-treatment sera of British patients with invasive carcinoma and other malignancies (mainly genital), and their matched controls

DIAGNOSIS	Inv. Ca Untreated	Adeno. Ca. of cervix	Endometrial Cancer	Carcinoma of ovary	Carcinoma of vulva
PATIENTS	101.7 (29)	88.04 (7)	95.12 (10)	93.01 (5)	127.0 (3)
CONTROLS	83.2 (27)	108.07 (5)	93.68 (8)	61.11 (4)	84.68 (3)
PROBABILITY	P<.01	P>.05	P>.05	P>.05	P>.05

RESULTS. PART VI.

ON SERIAL SAMPLES.

IgA ANTIBODIES TO MEMBRANE ANTIGENS AND VIRUS CAPSID ANTIGENS OF HSV-2 INFECTED CELLS, IN PATIENTS WITH GENITAL HERPES.

PART VI. ON SERIAL SAMPLES

IgA ANTIBODIES TO MEMBRANE ANTIGENS AND VIRUS CAPSID ANTIGENS OF HSV-2 INFECTED CELLS, IN PATIENTS WITH GENITAL HERPES.

IgA anti-VCA (virus capsid antigen) antibodies and IgA anti-MA antibodies to HSV-2 infected cells in serial samples of serum from cases of genital herpes

Figure 6.6 is a representation on a time scale of the presence of IgA anti-MA antibodies and IgA anti-VCA antibodies in serial samples of serum from 16 patients with genital herpes. In each case the first serum was collected within one to two months of a clinical attack during which HSV-2 virus was isolated from the cervix or vulva.

IgA anti-VCA was found to be transient, i.e. present for two to three months after clinical infection except in patient number 4 who continued to have these antibodies 10 months after the first clinical attack, and patient number 8 in whom IgA anti-VCA was first detected one year and three months after the initial clinical infection. These two patients had no recurrent attacks which were clinically apparent or which caused symptoms during this period. Also during this time attempts to isolate virus from these patients was unsuccessful.

In contrast, IgA anti-MA was found in every serial sample of serumfor the duration of follow up studies in all patients. This extended to one year and three months in patients numbers 7, 8 and 9. These patients also had no clinical attacks during this period.

IgA anti-VCA antibodies in serial samples of serum from 11 patients with abnormal cervical cytology.

Figure 6.7 is a representation on a time scale of the presence or absence of IgA anti-VCA and IgA anti-MA antibodies in serial samples of serum from 11 patients with abnormal cervical cytology. All but two (No's 4 and 9) of these 11 patients possessed IgA anti-VCA antibodies in their first serum samples.

IgA anti-VCA antibodies were found to persist in 5/9 patients (No's 2,3,5,6 and 11) for a period ranging from four months to 9 months. This is longer than in the 15/16 patients with genital herpes (Figure 6.9).

The two patients who were seronegative for IgA anti-VCA antibodies in their first serum samples did not have these antibodies in subsequent samples.

Age analysis of 20 British patients who presented with their first clinical attack of genital herpes.

The age range of these 20 patients was 18 - 39 years. The average age was 23.5 and the median 21.5.

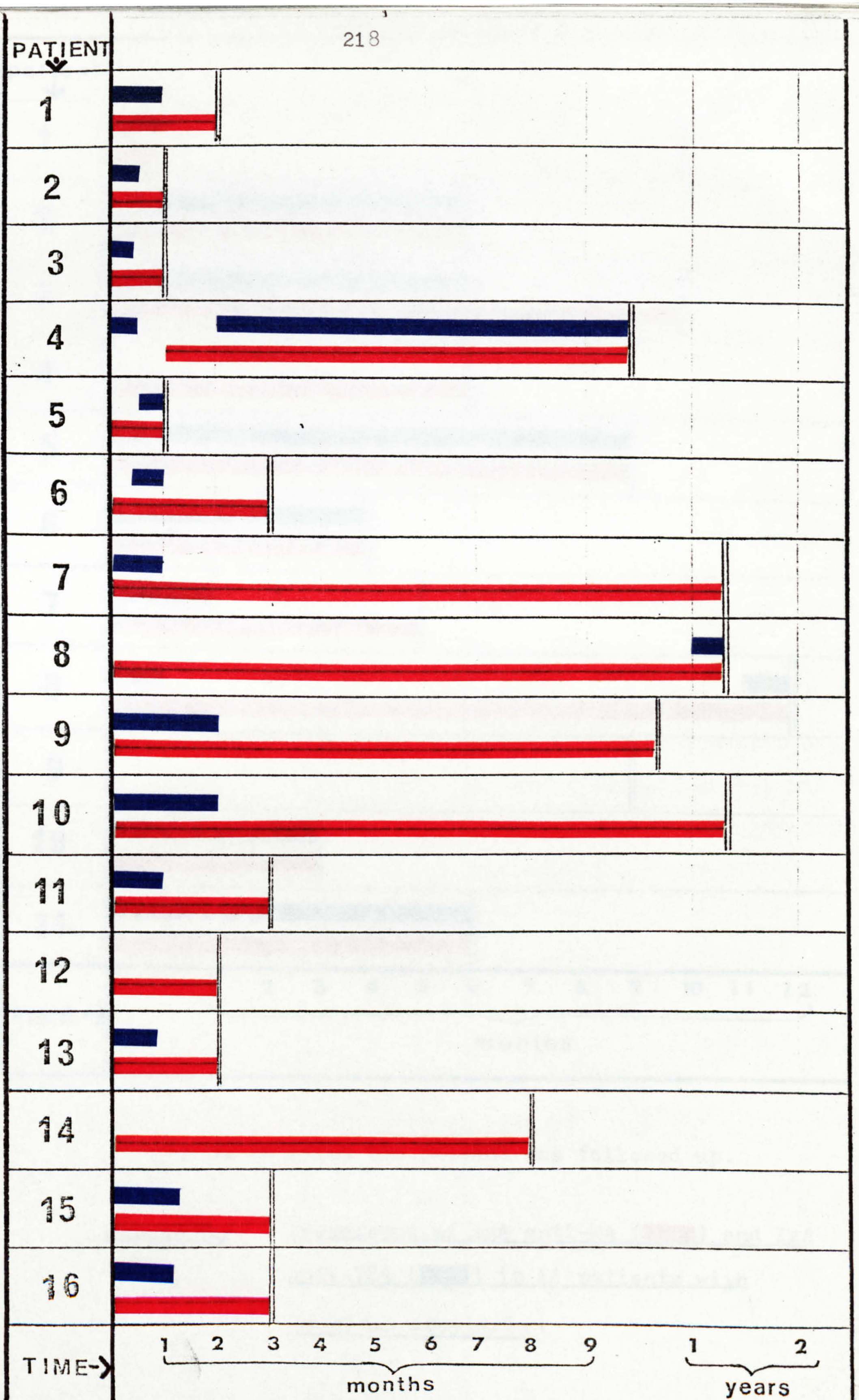
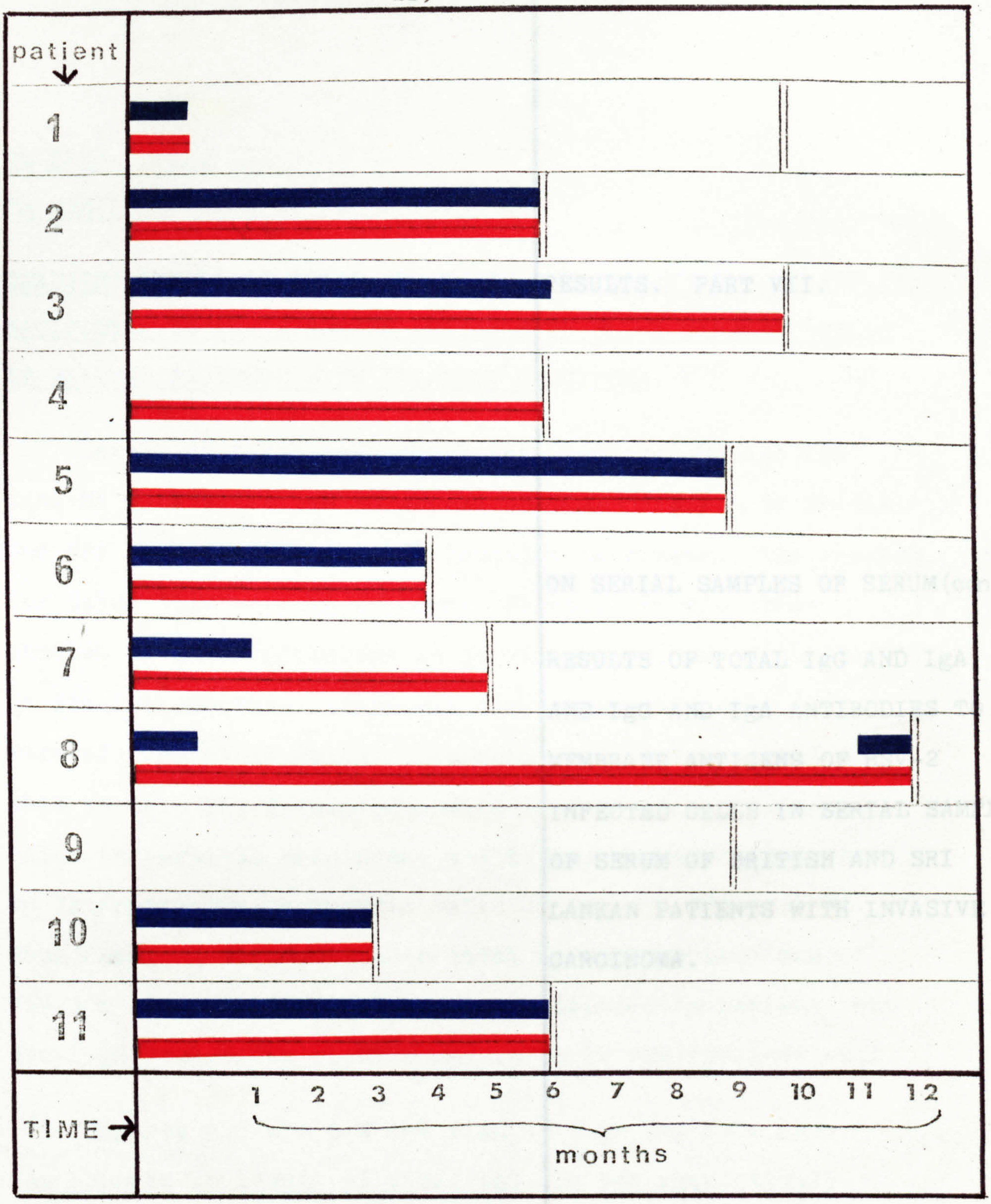


Figure 6.6 Prevalence of IgA anti-MA, ( ) and IgA anti-VCA, ( ) in 16 patients with genital herpes.

PERIOD OF TIME UP TO WHICH THE PATIENT WAS



‖ Time up to which the patient was followed up.

Figure 6.7 Prevalence of IgA anti-MA ( ) and IgA anti-VCA ( ) in 11 patients with cervical anaplasia

RESULTS. PART VII.

ON SERIAL SAMPLES OF SERUM (cont'd

RESULTS OF TOTAL IgG AND IgA, AND IgG AND IgA ANTIBODIES TO MEMBRANE ANTIGENS OF HSV-2 INFECTED CELLS IN SERIAL SAMPLES OF SERUM OF BRITISH AND SRI LANKAN PATIENTS WITH INVASIVE CARCINOMA.

RESULTS. PART VII.

ON SERIAL SAMPLES OF SERUM (cont'd)

CHANGING LEVELS OF TOTAL SERUM IgG AND IgA AND OF IgG AND IgA ANTIBODIES TO THE MEMBRANE ANTIGENS OF HSV-2 INFECTED CELLS IN BRITISH PATIENTS WITH INVASIVE CARCINOMA.

Levels of total IgG, total IgA, IgG anti-MA and IgA anti-MA were estimated in serial samples of serum of British and Sri Lankan patients with invasive carcinoma. The reasons for doing this were as follows: Firstly, in the three studies on immunoglobulins in cervical carcinoma described in the introduction, there was some suggestion that they were of prognostic value. Secondly, it has been claimed that certain HSV-2 related antibodies are also of prognostic value in cervical carcinoma, and it was therefore important to determine the prognostic value of IgG and IgA anti-MA. Thus changing trends of both total immunoglobulins and of HSV-2 related antibodies have been compared in patients who developed tumor recurrences and in those who remained well.

Figures 6.8 and 6.9 and figures 6.10 and 6.11 show the changes in levels of total IgG and IgA respectively among British patients, as determined by single radial immunodiffusion and plotted on a time scale. The number on each plot corresponds to the patient number on Tables 6.35 and 6.38. A change of 10% or more above or below the previous serum level was considered a rise (R) or fall(F). A change of less than 10% was classified as no change (NC).

The course of radiotherapy usually lasted for seven to

eight weeks. It was therefore assumed that by 10 weeks from the onset of treatment, any alteration in immunoglobulin levels or antibody titres which might have resulted from the effects of radiotherapy would by then have been stabilised. Thus the changes were assessed at two stages during the period of follow up.

a) At 10 weeks from the onset of treatment, and

b) At the end of the period of follow up.

The overall changes up to the end of the period of follow up has been referred to as gross change.

Method of determining gross change.

The gross change up to the time of collecting the last serum was determined as follows.

a) A fall in level of immunoglobulin or titre of antibody up to a period of 10 weeks from the onset of treatment which persisted at this level in subsequent sera was considered a gross fall (patient No. 10 on Table 6.35).

b) A rise in level of immunoglobulin or titre of antibody up to a period of 10 weeks from the onset of treatment which persisted at this level in subsequent sera was considered a gross rise (patient No. 14 on Table 6.38).

c) A rise in level of immunoglobulin or titre of antibody up to 10 weeks from the onset of treatment followed by a fall thereafter was considered a gross fall, i.e. the patient was beginning to demonstrate a falling trend in the level of immunoglobulin or in titre of antibody (patient No. 7 on Table 6.35).

d) Similarly a fall in level of immunoglobulin or titre

of antibody up to a period of 10 weeks from the onset of treatment followed by a rise was considered a gross rise, i.e. the patient was beginning to show a rising trend in the level of immunoglobulin or the titre of antibody (patient No. 22 on Table 6.35).

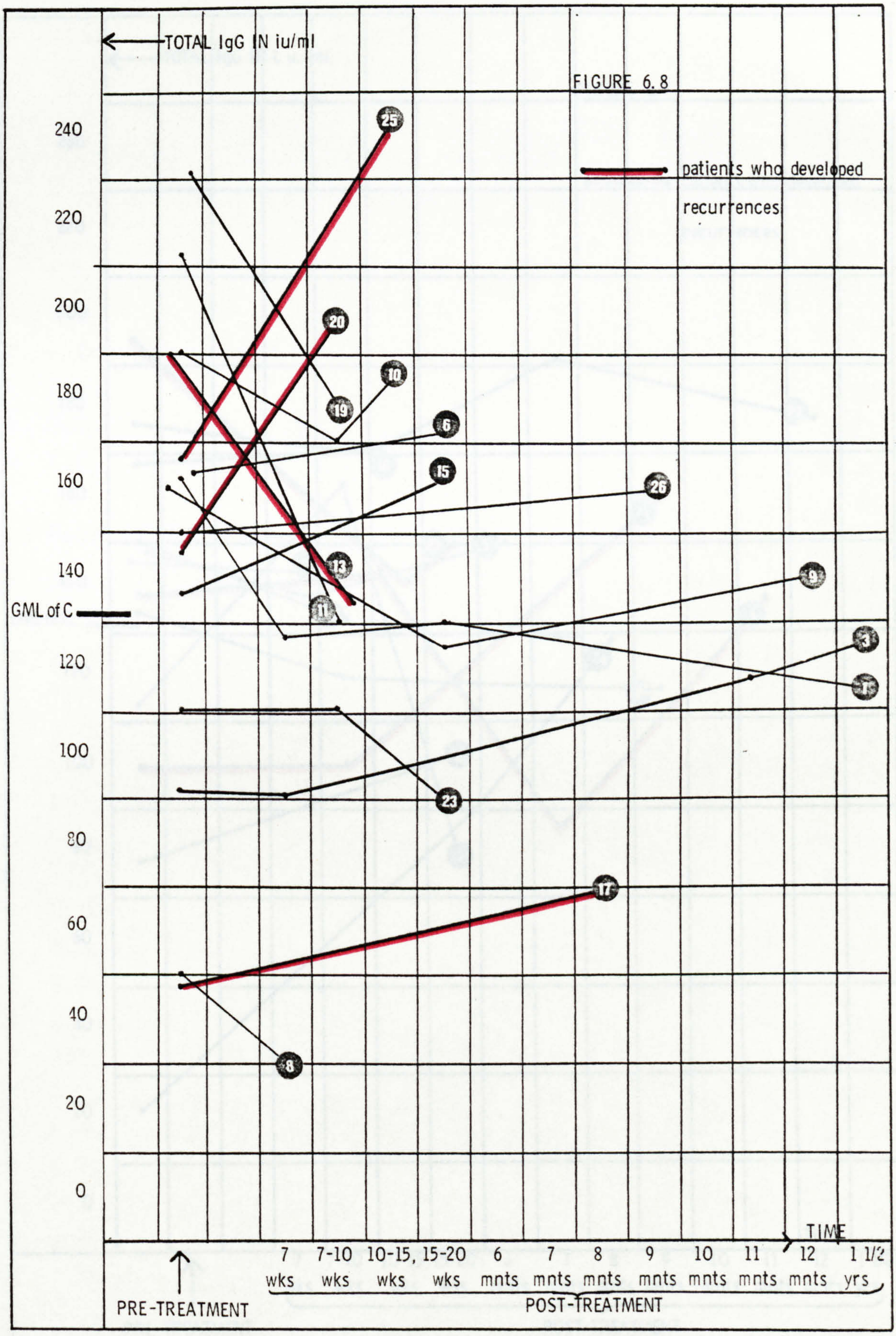
CHANGES IN LEVEL OF TOTAL IgG

Rising levels of total IgG tended to be associated with recurrences. Thus by the end of 10 weeks from the onset of treatment 3 of 7 (42%) patients who developed recurrences showed rising levels, in comparison with 4 of 20 (20%) patients who have remained well ($P > .05$) (Table 6.36). This association was even more striking when sera collected after 10 weeks from the onset of treatment were tested since 5 of 5 (100%) patients who developed recurrences demonstrated rising levels in contrast with 5 of 15 (33%) who remained well ($P < .01$) (Table 6.37).

CHANGES IN LEVEL OF TOTAL IgA

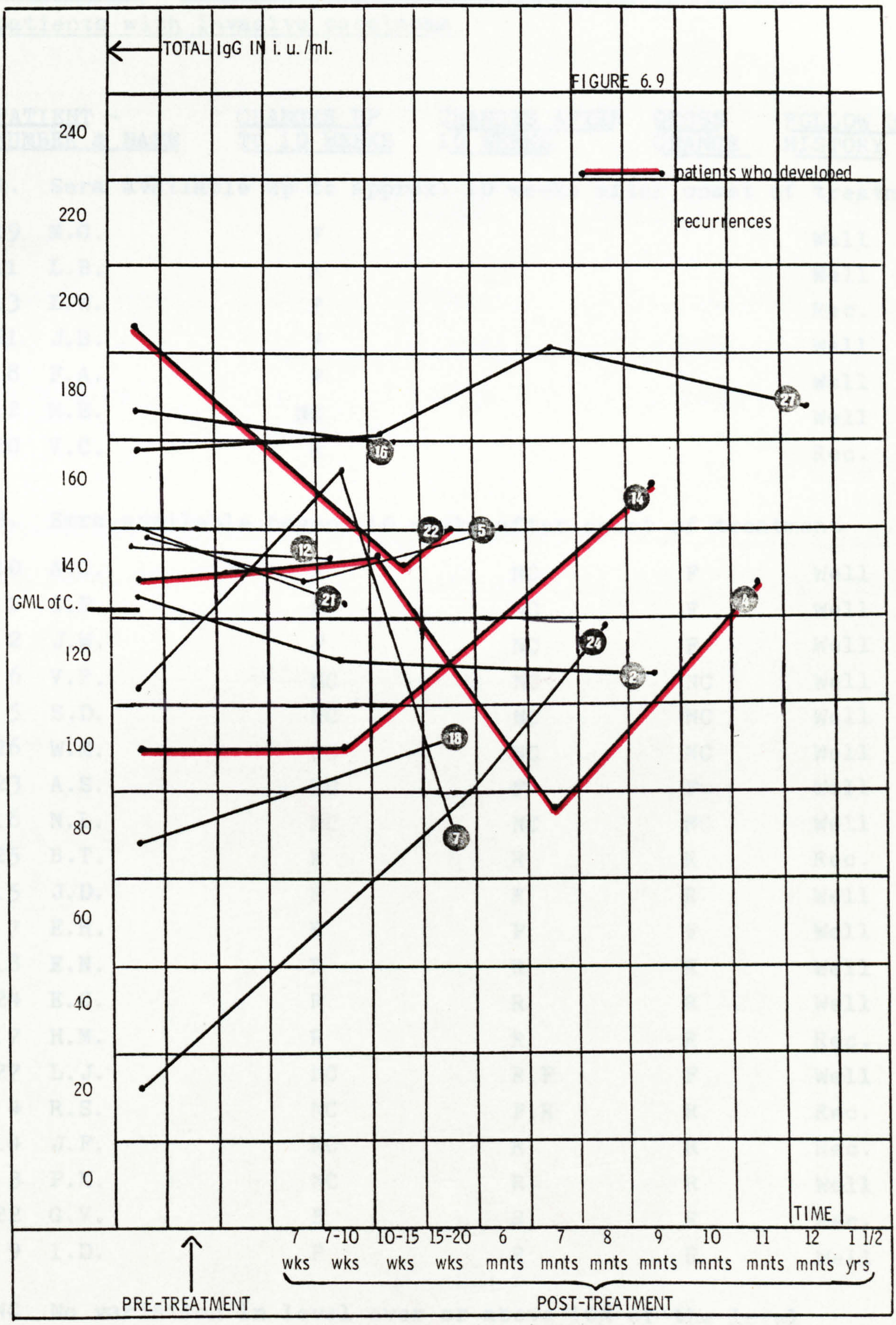
The association between a rise in total IgA and tumor recurrences was less clearcut than between total IgG and tumor recurrences. Thus up to 10 weeks following treatment there was no significant difference in changes in IgA level in patients who developed recurrences and those who remained well (Table 6.39). However after 10 weeks from the onset of treatment, 4 of 5 (30%) patients who developed recurrences demonstrated a rise in level. This contrasts with 6 of 15 (40%) who remained well ($P > .05$) (Table 6.40).

Figure 6.8 Changes in level of Total IgG in 15 British patients with invasive carcinoma, represented on a time scale.



GML of C - The geometric mean level of Total IgG in controls

Figure 6.9 Changes in level of Total IgG in 12 British patients with invasive carcinoma, represented on a time scale.



GML of C - The geometric mean level of Total IgG in controls

Table 6.35 Changes in level of IgG observed in British patients with invasive carcinoma

PATIENT - NUMBER & NAME		CHANGES UP TO 10 WEEKS	CHANGES AFTER 10 WEEKS	GROSS CHANGE	FOLLOW UP HISTORY
A. Sera available up to approx. 10 weeks after onset of treatment					
19	M.G.	F			Well
11	L.B.	F			Well
13	L.W.	F			Rec.
21	J.B.	F			Well
8	F.A.	F			Well
12	M.E.	NC			Well
20	V.C.	R			Rec.
B. Sera available beyond 10 weeks after onset of treatment					
10	A.G.	F	NC	F	Well
1	G.P.	F	NC	F	Well
2	J.W.	F	NC	F	Well
6	V.F.	NC	NC	NC	Well
5	S.D.	NC	NC	NC	Well
26	W.M.	NC	NC	NC	Well
23	A.S.	NC	F	F	Well
16	N.L.	NC	NC	NC	Well
25	B.T.	R	R	R	Rec.
15	J.D.	R	R	R	Well
7	E.H.	R	F	F	Well
18	E.N.	R	R	R	Well
24	E.C.	R	R	R	Well
17	H.M.	R	R	R	Rec.
27	L.J.	NC	R,F	F	Well
4	R.S.	NC	F,R	R	Rec.
14	J.F.	NC	R	R	Rec.
3	P.K.	NC	R	R	Well
22	G.V.	F	R	R	Rec.
9	I.D.	F	R	R	Well

NC No variation in level over or above 10% of the level observed in the previous serum.

F Fall in level over 10% of that observed in previous serum.

R Rise in level over 10% of that observed in previous serum.

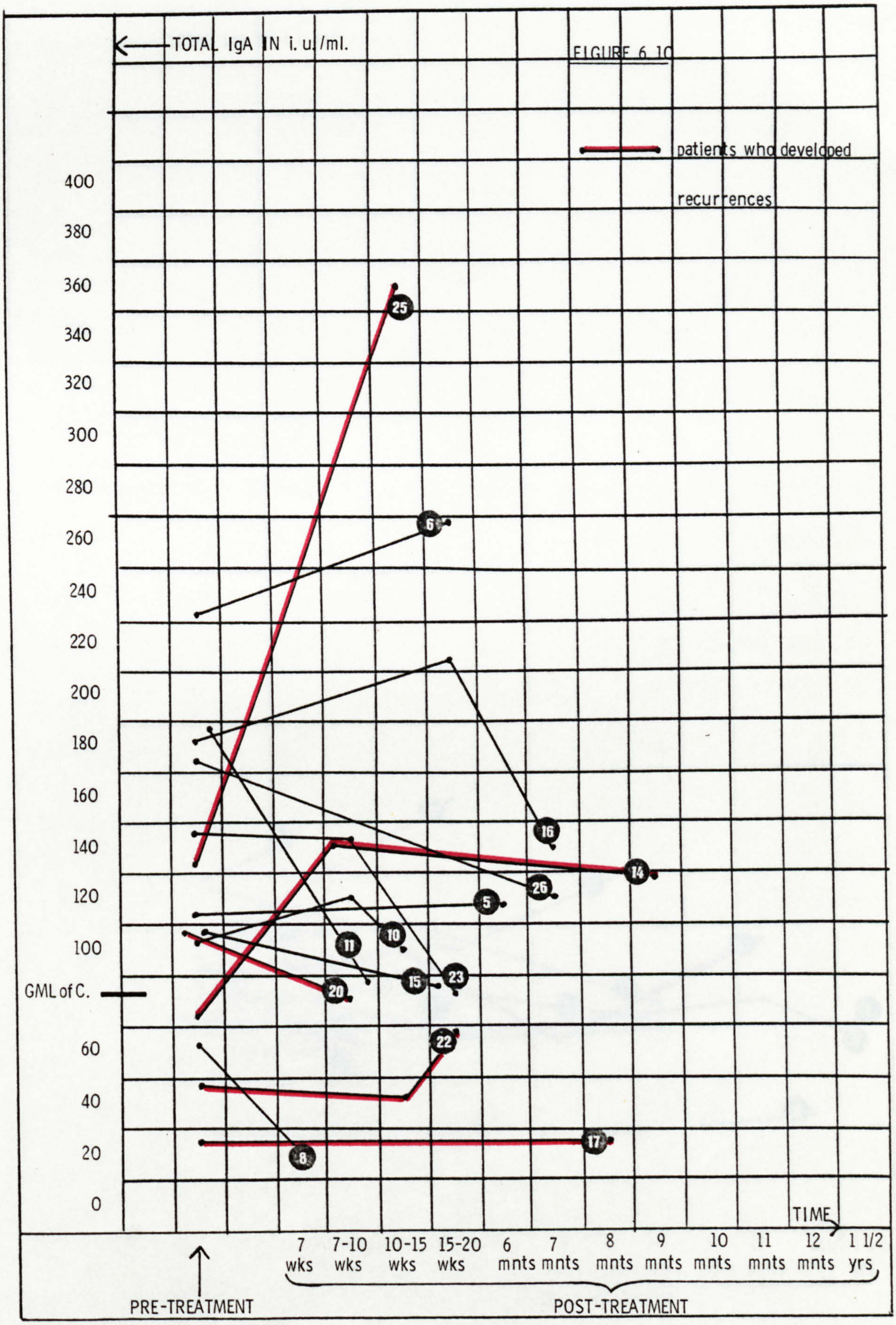
Table 6.36 Changes in level of total IgG up to 10 weeks after the onset of treatment, in British patients with invasive carcinoma.

	TOTAL	RECURRENCE PATIENTS	WELL PATIENTS	P_2 (X^2-2 tailed)
Total	27	7	20	
Rise in level	7	3 (42%)	4 (20%)	> .05
Fall in level	10	2 (29%)	8 (40%)	
Unchanging levels	10	2 (29%)	8 (40%)	

Table 6.37 Changes in levels of total IgG after 10 weeks from the onset of treatment, in British patients with invasive carcinoma.

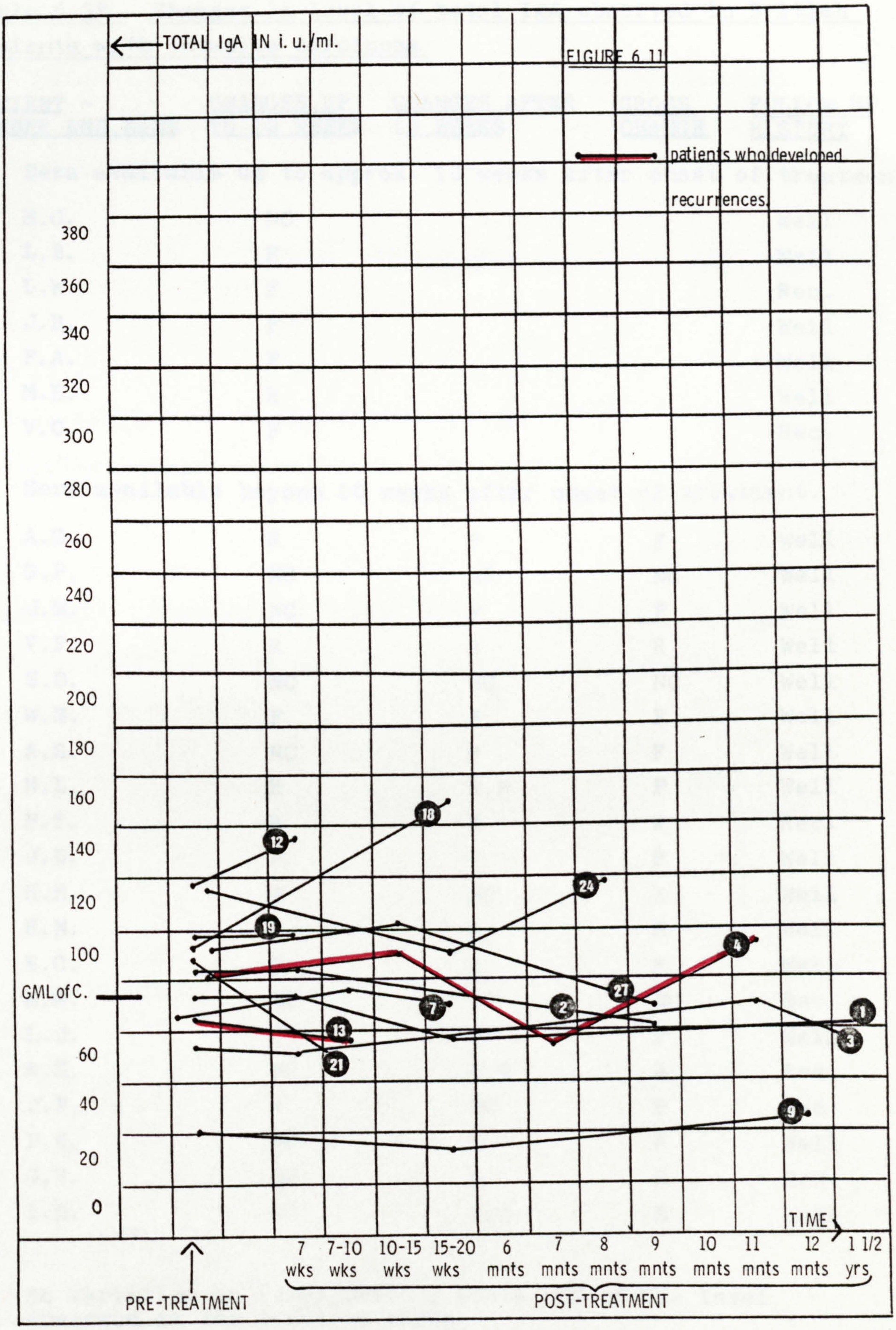
	TOTAL	RECURRENCE PATIENTS	WELL PATIENTS	P_2 (X^2 - 2 tailed)
Total	20	5	15	
Rise in level	10	5 (100%)	5 (33%)	< .01
Fall in level	6	0	6	
Unchanging levels	4	0	4	

Figure 6.10 Changes in level of Total IgA in 14 British patients with invasive carcinoma, represented on a time scale.



GML of C - The geometric mean level of Total IgA in controls

Figure 6.11 Changes in level of Total IgA in 13 British patients with invasive carcinoma, represented on a time scale.



GML of C - The geometric mean level of Total IgA in controls

Table 6.38 Changes in level of total IgA observed in British patients with invasive carcinoma

PATIENT - NUMBER AND NAME		CHANGES UP TO 10 WEEKS	CHANGES AFTER 10 WEEKS	GROSS CHANGE	FOLLOW UP HISTORY
A. Sera available up to approx. 10 weeks after onset of treatment.					
19	M.G.	NC			Well
11	L.B.	F			Well
13	L.W.	F			Rec.
21	J.B.	F			Well
8	F.A.	F			Well
12	M.E.	R			Well
20	V.C.	F			Rec.
B. Sera available beyond 10 weeks after onset of treatment.					
10	A.G.	R	F	F	Well
1	G.P.	NC	NC	NC	Well
2	J.W.	NC	F	F	Well
6	V.F.	R	R	R	Well
5	S.D.	NC	NC	NC	Well
26	W.M.	F	F	F	Well
23	A.S.	NC	F	F	Well
16	N.L.	R	R,F	F	Well
25	B.T.	R	R	R	Rec.
15	J.D.	F	F	F	Well
7	E.H.	R	NC	R	Well
18	E.N.	R	R	R	Well
24	E.C.	F	R	R	Well
17	H.M.	NC	NC	NC	Rec.
27	L.J.	NC	F	F	Well
4	R.S.	NC	F,R	R	Rec.
14	J.F.	R	NC	R	Rec.
3	P.K.	NC	R	R	Well
22	G.V.	NC	R	R	Rec.
9	I.D.	NC	F,R	R	Well

NC No variation in level over or above 10% of the level observed in the previous serum.

F Fall in level over 10% of that observed in previous serum.

R Rise in level over 10% of that observed in previous serum.

Table 6.39 Changes in levels of total IgA up to 10 weeks after the onset of treatment, in patients with invasive carcinoma from Britain

	TOTAL	RECURRENCE PATIENTS	WELL PATIENTS
Total	27	7	20
Rise in level	8	2 (29%)	6 (30%)
Fall in level	8	2 (29%)	6 (30%)
Unchanging levels	11	3 (42%)	8 (40%)

Table 6.40 Changes in levels of total IgA after 10 weeks from the onset of treatment in patients with invasive carcinoma from Britain

	TOTAL	RECURRENCE PATIENTS	WELL PATIENTS	P (X^2 2-tailed)
Total	20	5	15	
Rise in level	10	4 (80%)	6 (40%)	$>.05$
Fall in level	7	0	7	
Unchanging levels	3	1	2	

It is noteworthy that none of the patients who developed recurrences demonstrated a gross fall in level of total IgG or total IgA.

CHANGES IN TITRE OF IgG AND IgA ANTIBODY TO MEMBRANE ANTIGENS (MA) OF HSV-2 INFECTED CELLS, IN SERIAL SAMPLES OF SERUM FROM BRITISH PATIENTS WITH INVASIVE CARCINOMA.

Figures 6.12 and 6.13 and figure 6.14 show the changes in titre of IgG anti-MA and IgA anti-MA respectively in serial samples of serum from British patients with invasive carcinoma, plotted against a time scale. The number on each plot corresponds to the patient number in Table 6.41 and Table 6.44

CHANGES IN TITRE OF IgG ANTI-MA

Rising titres of IgG anti-MA were associated with recurrences. Although this was observed in sera collected up to a period of 10 weeks from the onset of treatment, after this interval there was a greater difference in the incidence of rising titres among patients with recurrences than those who remained well. Thus up to 10 weeks from the onset of treatment, 6 of 7 (86%) patients who developed recurrences demonstrated rising titres of IgG anti-MA in comparison with 11 of 20 (55%) of patients who remained well ($P > .05$)(Table 6.42). In the sera collected after this period, 4 of 5 (80%) patients who developed recurrences had rising titres in contrast with 5 of 15 (33%) of the patients who remained well ($P < .05$) (Table 6.43).

However it must be emphasised that these rising titres were not dramatic ones; often the rise in titre was only a two- to four-fold difference. However on testing these sera repeatedly and testing them under code these differences were consistently detected.

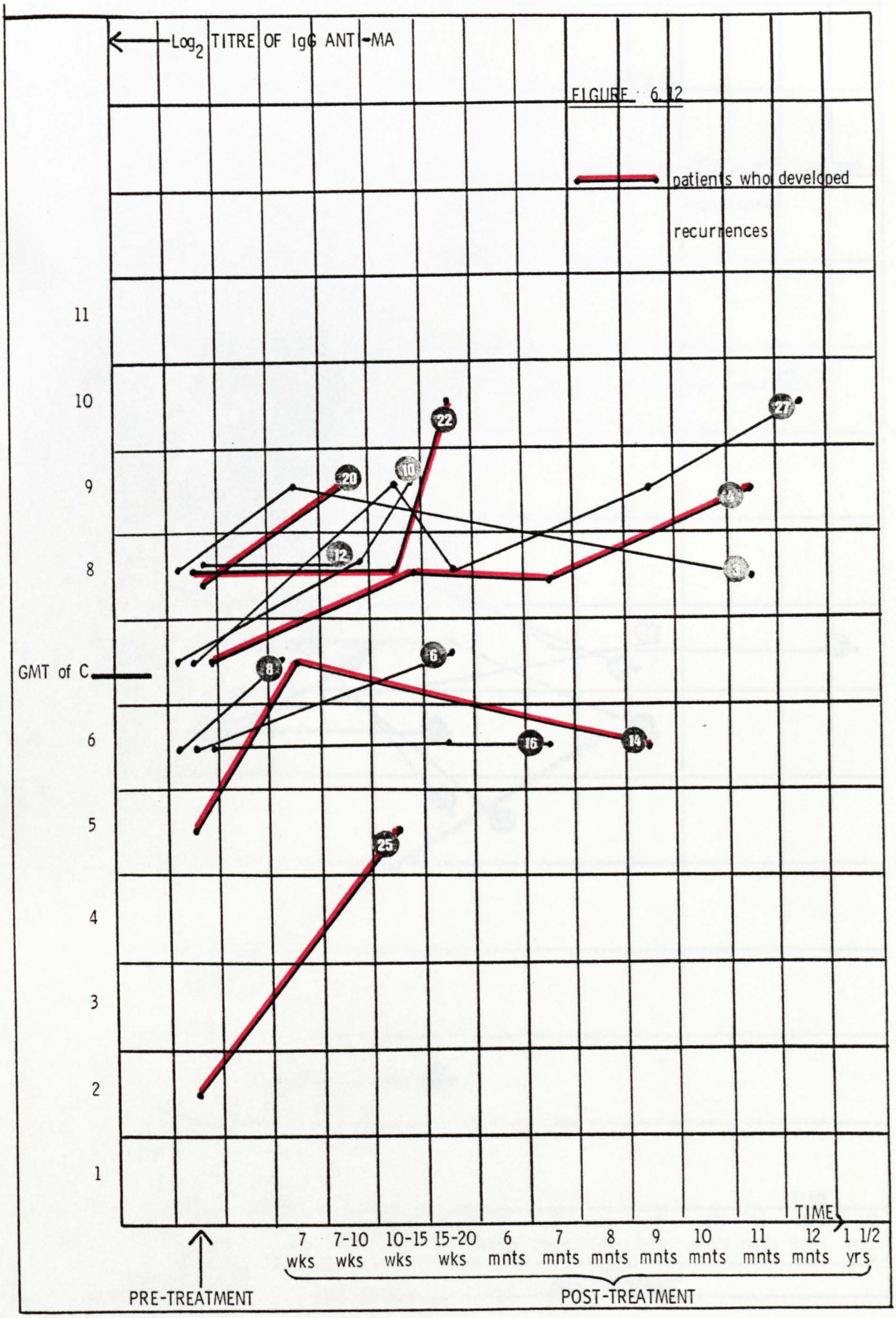
CHANGES IN TITRE OF IgA ANTI-MA

In pre-treatment serum samples it was IgA anti-MA titres that clearly distinguished patients with invasive carcinoma from controls. These titres also increased with the stage of disease. Despite this, attempts to detect trends of changing levels of IgA anti-MA did not help in distinguishing between patients who developed recurrences and those who remained well. This is seen from Table 6.45 which shows the changes up to 10 weeks from the onset of treatment and Table 6.46 which shows the gross changes in titre thereafter. It is however relevant to other findings in this study which are discussed later that of the patients who remained well, 9 of 20 (45%) showed a fall in titre of IgA anti-MA up to 10 weeks from the onset of treatment but only 4 of 15 (27%) showed a fall in titre by the end of the period of follow up, i.e. in some of the patients who remained well, the IgA anti-MA titres had reverted to their original levels.

SUMMARY OF THE GROSS CHANGES IN TOTAL IgG AND IgA ANTI-MA IN BRITISH PATIENTS WITH INVASIVE CARCINOMA

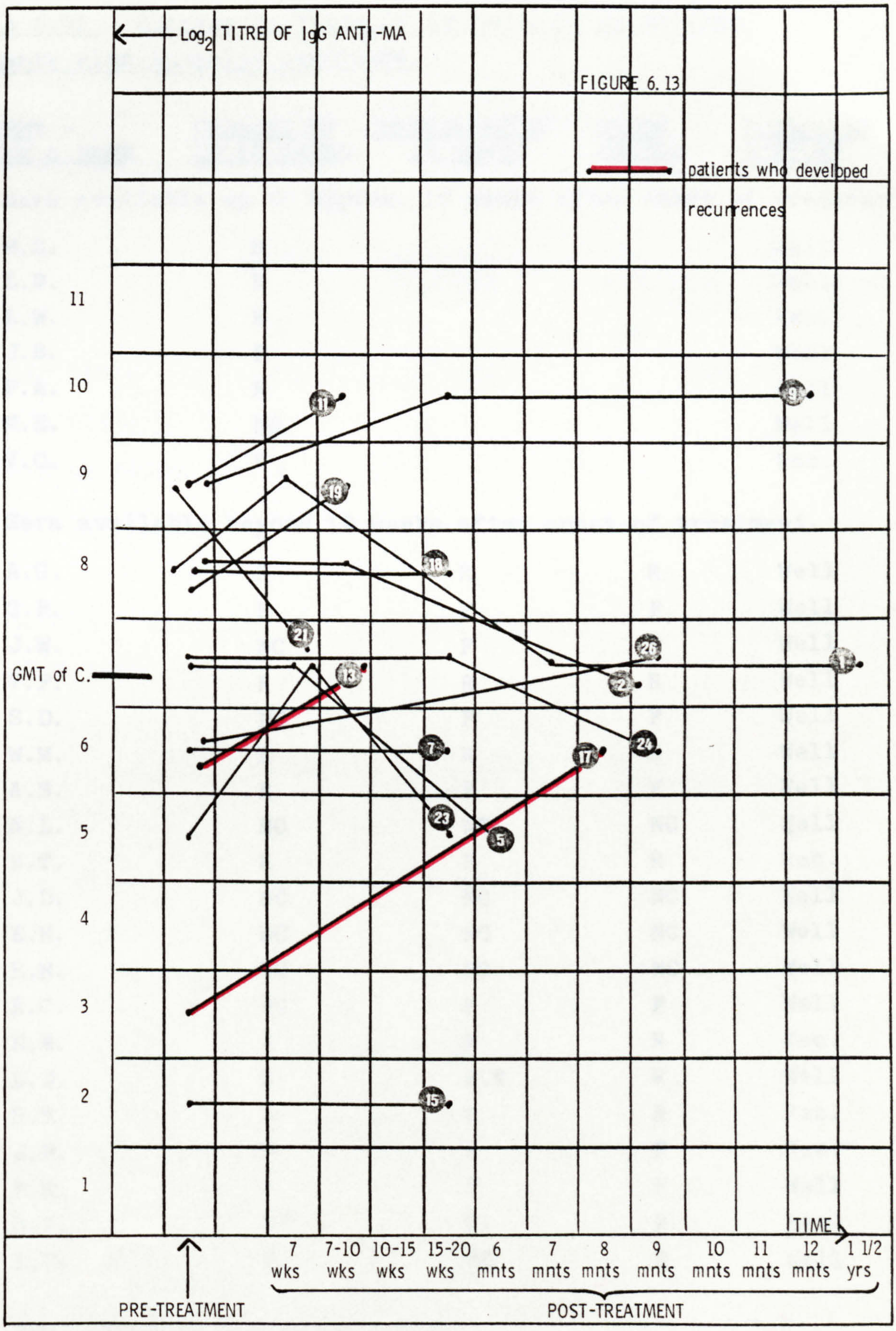
Table 6.47A summarises details of patients who developed recurrences and those who remained well or who showed

Figure 6.12 Changes in titre of IgG anti-MA in 12 British patients with invasive carcinoma, represented on a time scale.



GMT of C - GMT of IgG anti-MA in controls

Figure 6.13 Changes in titre of IgG anti-MA in 15 British patients with invasive carcinoma, represented on a time scale.



GMT of C - GMT of IgG anti-MA in controls

Table 6.41 Changes in titre of IgG anti-MA in British patients with invasive carcinoma.

PATIENT - NUMBER & NAME		CHANGES UP TO 10 WEEKS	CHANGES AFTER 10 WEEKS	GROSS CHANGE	FOLLOW UP HISTORY
A. Sera available up to approx. 10 weeks after onset of treatment.					
19	M.G.	R			Well
11	L.B.	R			Well
13	L.W.	R			Rec.
21	J.B.	F			Well
8	F.A.	R			Well
12	M.E.	NC			Well
20	V.C.	R			Rec.
B. Sera available beyond 10 weeks after onset of treatment.					
10	A.G.	R	R	R	Well
1	G.P.	R	F	F	Well
2	J.W.	NC	F	F	Well
6	V.F.	R	R	R	Well
5	S.D.	NC	F	F	Well
26	W.M.	R	R	R	Well
23	A.S.	R	F	F	Well
16	N.L.	NC	NC	NC	Well
25	B.T.	R	R	R	Rec.
15	J.D.	NC	NC	NC	Well
7	E.H.	NC	NC	NC	Well
18	E.N.	NC	NC	NC	Well
24	E.C.	NC	F	F	Well
17	H.M.	R	R	R	Rec.
27	L.J.	R	F.R	R	Well
4	R.S.	R	R	R	Rec.
14	J.F.	R	F	F	Rec.
3	P.K.	R	F	F	Well
22	G.V.	NC	R	R	Rec.
9	I.D.	R	NC	R	Well

NC Same titre as the serum before.
F Fall in titre (2-fold fall in titre inclusive).
R Rise in titre (2-fold rise in titre inclusive).

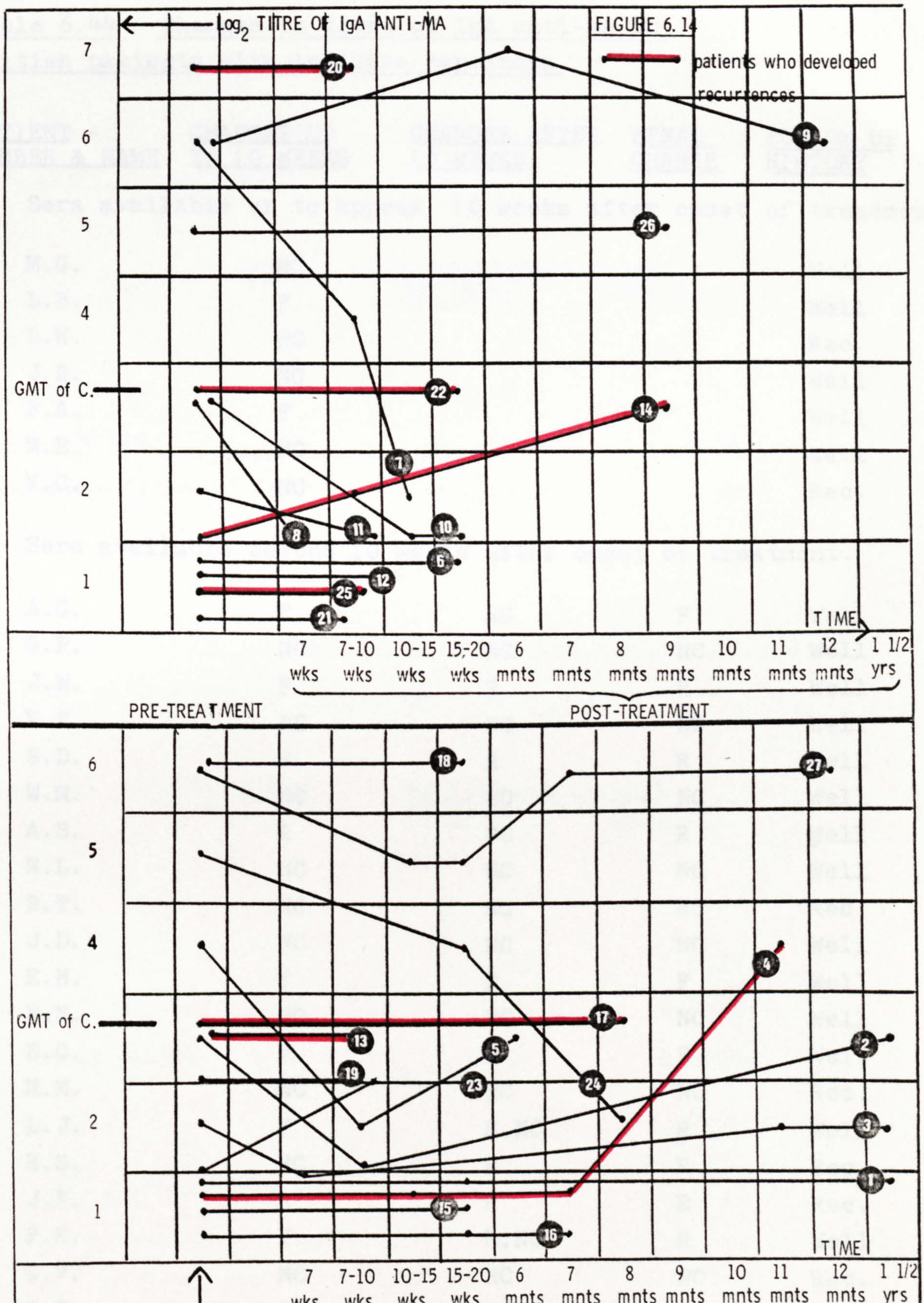
Table 6.42 Changes in titre of IgG anti-MA up to 10 weeks after the onset of treatment in British patients with invasive carcinoma

	TOTAL	RECURRENCE PATIENTS	WELL PATIENTS	P_2 (X^2- 2 tailed)
Total	27	7	20	
Rise in titre	17	6 (86%)	11 (55%)	>.05
Fall in titre	1	0	1	
Unchanging titre	9	1	8	

Table 6.43 Gross changes in titre of IgG anti-MA in British patients with invasive carcinoma

	TOTAL	RECURRENCE PATIENTS	WELL PATIENTS	P_2 (X^2- 2 tailed)
Total	20	5	15	
Rise in titre	9	4 (80%)	5 (33%)	<.05
Fall in titre	7	1	6	
Unchanging levels	4	0	4	

Figure 6.14 Changes in titre of IgA anti-MA in 27 British patients with invasive carcinoma, represented on a time scale.



GMT of C - GMT of IgA anti-MA in controls

Table 6.44 Changes in titre of IgA anti-MA in British patients with invasive carcinoma.

PATIENT - NUMBER & NAME		CHANGES UP TO 10 WEEKS	CHANGES AFTER 10 WEEKS	FINAL CHANGE	FOLLOW UP HISTORY
A. Sera available up to approx. 10 weeks after onset of treatment.					
19	M.G.	NC			Well
11	L.B.	F			Well
13	L.W.	NC			Rec.
21	J.B.	NC			Well
8	F.A.	F			Well
12	M.E.	NC			Well
20	V.C.	NC			Rec.
B. Sera available beyond 10 weeks after onset of treatment.					
10	A.G.	F	NC	F	Well
1	G.P.	NC	NC	NC	Well
2	J.W.	F	R	R	Well
6	V.F.	NC	NC	NC	Well
5	S.D.	F	R	R	Well
26	W.M.	NC	NC	NC	Well
23	A.S.	R	NC	R	Well
16	N.L.	NC	NC	NC	Well
25	B.T.	NC	NC	NC	Rec.
15	J.D.	NC	NC	NC	Well
7	E.H.	F	F	F	Well
18	E.N.	NC	NC	NC	Well
24	E.C.	F	F	F	Well
17	H.M.	NC	NC	NC	Rec.
27	L.J.	F	R,NC	R	Well
4	R.S.	NC	R	R	Rec.
14	J.F.	R	R	R	Rec.
3	P.K.	F	R,NC	R	Well
22	G.V.	NC	NC	NC	Rec.
9	I.D.	R	F	F	Well

NC Same titre as the serum before.
F Fall in titre (2-fold fall in titre inclusive).
R Rise in titre (2-fold rise in titre inclusive).

Table 6.45 Changes in titre of IgA anti-MA up to 10 weeks after the onset of treatment in British patients with invasive carcinoma

	TOTAL	RECURRENCE PATIENTS	WELL PATIENTS	
Total	27	7	20	
Rise in titre	3	1 (14%)	2 (10%)	
Fall in titre	9	0	9 (45%)	90%
Unchanging titre	15	6 (86%)	9 (45%)	

Table 6.46 Gross changes in titre of IgA anti-MA in British patients with invasive carcinoma

	TOTAL	RECURRENCE PATIENTS	WELL PATIENTS
Total	20	5	15
Rise in titre	7	2 (40%)	5 (33%)
Fall in titre	4	0	4 (27%)
Unchanging titre	9	3 (60%)	6 (40%)

Table 6.47 Gross change in IgG anti-MA and total immunoglobulin observed in British patients with invasive carcinoma

A.

	RISE IN IgG ANTI-MA	RISE IN TOTAL IgG	RISE IN TOTAL IgA
Recurrence patients	4/5 (80%)	5/5 (100%)	4/5 (80%)
Well patients	5/15 (33%)	5/15 (33%)	6/15 (40%)

B.

	↑IgG ANTI-MA ↑TOTAL IgA	↑IgG ANTI-MA ↑TOT. IgG	↑TOT. IgG ↑TOT. IgA	↑IgG ANTI-MA ↑TOTAL IgG ↑TOTAL IgA
Recurrence patients	3/5 (60%)	4/5 (80%)	4/5 (80%)	3/5 (60%)
Well patients	2/15 (13%)	1/15 (6%)	4/15 (26%)	1/15 (6%)

* P .0005 (X^2 - 2 tailed)

↑ rise in

a) a rise in total IgG, b) a rise in total IgA and c) a rise in IgG anti-MA.

In Table 6.47B the proportion of these two groups of patients who showed all possible combinations of the changes discussed above have been examined. A combination of a rise of IgG anti-MA and rise in total IgG gave the best distinction between patients who developed recurrences and those who remained well.

CHANGES IN LEVELS OF TOTAL IgG AND IgA ANTIBODY TO THE MEMBRANE ANTIGENS OF HSV-2 INFECTED CELLS IN SRI LANKAN PATIENTS WITH CERVICAL CARCINOMA - A COMPARISON WITH RESULTS ON BRITISH PATIENTS WITH INVASIVE CARCINOMA

The changes in immunoglobulin levels and antibody titres in Sri Lankan patients with invasive carcinoma confirmed the results observed among British patients. Follow up histories were available on 20 of 29 Sri Lankan patients from whom serial samples were obtained. From 12 of these 20 patients, serial samples were obtained up to a period of 10 weeks from the onset of treatment and from eight up to a period of approximately 20 weeks. Unfortunately six patients who developed recurrences were among the 12 from whom sera were collected for an interval of 10 weeks from the onset of treatment. Therefore a study of how well changes in level of immunoglobulin and titre of antibody distinguished patients who developed recurrences from those who remained well, could be done only on sera collected up to a period of 10 weeks from the onset of treatment.

These results have been compared below to the results on British sera collected up to the same period.

Later on page 250 changes in level of immunoglobulin and titre of antibody have been compared between:

a) Eight Sri Lankan patients who did not develop recurrences and who were followed up for longer than 10 weeks from the onset of treatment, and

b) 15 British patients who similarly did not develop recurrences and who were followed up for a longer period than 10 weeks from the onset of treatment.

Tables 6.48, 6.49, 6.51 and 6.52 give details of the follow up histories on Sri Lankan patients and details of the changes observed in level of immunoglobulin and antibody titre as for British patients.

CHANGES IN LEVEL OF TOTAL IgG

Up to a period of 10 weeks from the onset of treatment (Table 6.50A) a rise in level of total IgG was seen more frequently in Sri Lankan patients who developed recurrences (4 of 6 = 67%) than among patients who remained well (3 of 13 = 23%) ($P < .05$). These results are simllar to those obtained in British patients with invasive carcinoma (Table 6.36).

CHANGES IN LEVEL OF TOTAL IgA

Up to a period of 10 weeks from the onset of treatment the changes in level of total IgA were similar among patients who developed recurrences and those who remained well. These results are compatible with those observed up to 10 weeks from

the onset of treatment in British patients with invasive carcinoma (Table 6.39).

CHANGES IN TITRE OF IgG ANTI-MA

Three of six Sri Lankan patients who developed recurrences had no detectable IgG anti-MA (Table 6.51). However of the three in whom IgG anti-MA was detected, two (66%) showed a rise in IgG anti-MA by 10 weeks from the onset of treatment. In contrast only 5 of 13 (38%) of patients who remained well showed a similar rise of IgG anti-MA (Table 6.53). The equivalent proportions among British patients was 86% and 55% repectively (Table 6.42).

CHANGES IN TITRE OF IgA ANTI-MA

Although a significantly higher proportion of Sri Lankan patients possessed IgA anti-MA in comparison with controls, 5 of 6 patients who developed recurrences had no detectable IgA anti-MA (Table 6.52). However 6 of 13 (46%) Sri Lankan patients who remained well showed a fall in titre of IgA anti-MA or had stable titres (Table 6.54). Among British patients this proportion was 9 of 20 (45%) (Table 6.45).

CHANGES AFTER 10 WEEKS FROM TREATMENT OF LEVELS OF TOTAL IgG AND TOTAL IgG ANTI-MA AND IgA ANTI-MA IN SRI LANKAN PATIENTS WITH INVASIVE CARCINOMA

Eight Sri Lankan patients from whom serial samples of serum were collected for a period of 20 weeks from the onset

Table 6.48 Changes in level of total IgG observed in Sri Lankan patients with invasive carcinoma

PATIENT - NUMBER & NAME		CHANGES UP TO 10 WEEKS	CHANGES UP TO 20 WEEKS		FOLLOW UP HISTORY
11	H.R.N.	R			Well
12	S.W.	F			Well
13	A.A.	NC			Well
28	J.M.J.	F			Well
31	K.A.B.	F	Sera not available after 10 weeks.		Well
7	W.C.H.	R			Rec.
9	M.G.R.	R			Rec.
17	V.deS.	F			Rec.
18	D.M.	R			Rec.
22	B.G.S.	NC			Terminal
23	M.E.	R			Rec.
26	K.R.	F			Rec.
1	P.D.	R	R	R	Well
6	D.A.	F	F	F	Well
8	U.G.P.	F	F	F	Well
15	P.N.	F	F	F	Well
19	K.T.N.	R	F	F	Well
20	A.D.	F	R	R	Well
25	S.H.	F	F	F	Well
30	M.A.	F	R	R	Well
3	W.L.	F	F	F	Not known
16	T.K.	F	F	F	
21	V.P.L.	NC			
24	M.M.	R			
27	G.G.R.	NC			
29	B.M.	R			
32	M.H.	NC			
2	P.D.	R	F	F	
10	J.R.	F	F	F	

NC No change.
F Fall in level over 10% of that observed in previous serum.
R Rise in level over 10% of that observed in previous serum.

Table 6.49 Changes in level of total IgA observed in Sri Lankan patients with invasive carcinoma

PATIENT - NUMBER & NAME		CHANGE UP TO 10 WEEKS	CHANGE UP TO 20 WEEKS		FOLLOW UP HISTORY
11	H.R.N.	NC			Well
12	S.W.	R			Well
13	A.A.	F			Well
28	J.M.J.	NC			Well
21	K.A.B.	NC			Well
7	W.C.H.	R	sera not available after 10 weeks		Rec.
9	M.G.R.	R			Rec.
17	V.deS.	NC			Rec.
18	D.M.	NC			Rec.
22	B.G.S.	NC			Terminal
23	M.E.	F			Rec.
26	K.R.	F			Rec.
1	P.D.	NC	NC	NC	Well
6	D.A.	NC	NC	NC	Well
8	U.G.P.	R	R	R	Well
15	P.N.	F	R	R	Well
19	K.T.N.	NC	F	F	Well
20	A.D.	NC	NC	NC	Well
25	S.H.	F	R	R	Well
30	M.A.	F	NC	F	Well
3	W.L.	F	—		Not known
16	T.K.	R			
21	V.P.L.	R			
24	M.M.	R			
27	G.G.R.	F			
29	B.M.	R			
32	M.H.	NC			
2	P.D.	R	F	F	
10	J.R.	F	F	F	

NC No change.
F Fall in level over 10% of that observed in previous serum.
R Rise in level over 10% of that observed in previous serum.

Table 6.50 Changes in level of total IgG and IgA up to 10 weeks from the onset of treatment in Sri Lankan patients with invasive carcinoma with known follow up histories

A. IgG

	TOTAL	RECURRENCE PATIENTS	TERMINAL CASES	WELL PATIENTS
Total	20	6	1	13
Rise in level	7	4 (67%)		3 (28%)
Fall in level	11	2 (33%)		9 (69%)
Unchanging levels	2	0	1	1

B. IgA

	TOTAL	RECURRENCE PATIENTS	TERMINAL CASES	WELL PATIENTS
Total	20	6	1	13
Rise in level	4	2 (33.3%)		2 (15%)
Fall in level	6	2 (33.3%)		4 (31%)
Unchanging levels	10	2 (33.3%)	1	7 (54%)

Table 6.51 Changes in titre of IgG anti-MA in 29 Sri Lankan patients with invasive carcinoma.

PATIENT - NUMBER & NAME	CHANGES UP TO 10 WEEKS	CHANGES UP TO 20 WEEKS	FINAL CHANGE	FOLLOW UP HISTORY
11 H.R.N.	F			Well
12 S.W.	R			Well
13 A.A.	R			Well
28 J.M.J.	R			Well
31 K.A.B.	NC	Sera not available		Well
7 W.C.H.	R	after 10 weeks.		Rec.
9 M.G.R.	NC			Rec.
17 V.deS.	R			Rec.
18 D.M.	NC (SN)			Rec.
22 B.G.S.	NC			Terminal
23 M.E.	NC (SN)			Rec.
26 K.R.	NC (SN)			Rec.
1 P.D.	R	NC	R	Well
6 D.A.	NC	NC	NC	Well
8 U.G.P.	NC	NC	NC	Well
15 P.N.	NC	NC	NC	Well
19 K.T.N.	NC	R	R	Well
20 A.D.	R	NC	R	Well
25 S.H.	NC	R	R	Well
30 M.A.	F	NC	F	Well
3 W.L.	R			Not known
16 T.K.	NC			
21 V.P.L.	NC			
24 M.M.	R			
27 G.G.R.	NC			
29 B.M.	NC			
32 M.H.	F			
2 P.D.	R	F	F	
10 J.R.	R	F	F	

SN Seronegative

NC No change in titre

F Fall in titre

R Rise in titre

Table 6.52 Changes in titre of IgA anti-MA in 29 Sri Lankan patients with invasive carcinoma.

PATIENT - NUMBER & NAME	CHANGES UP TO 10 WEEKS	CHANGES UP TO 20 WEEKS	FINAL CHANGE	FOLLOW UP HISTORY
11 H.R.N.	NC (SN)			Well
12 S.W.	F			Well
13 A.A.	NC			Well
28 J.M.J.	F			Well
31 K.A.B.	NC (SN)	Sera not available		Well
7 W.C.H.	NC (SN)	after 10 weeks		Rec.
9 M.G.R.	NC			Rec.
17 V.deS.	NC (SN)			Rec.
18 D.M.	NC (SN)			Rec.
22 B.G.S.	F			Terminal
23 M.E.	NC (SN)			Rec.
26 NC (SN)			Rec.	Rec.
1 P.D.	R	F	F	Well
6 D.A.	NC	NC	NC	Well
8 U.G.P.	NC	NC	NC	Well
15 P.N.	F	NC	F	Well
19 K.T.N.	NC	NC	NC	Well
20 A.D.	F	NC	F	Well
25 S.H.	F	F	F	Well
30 M.A.	F	NC	F	Well
3 W.L.	NC			Not known
16 T.K.	NC			
21 V.P.L.	R			
24 M.M.	NC			
27 G.G.R.	F			
29 B.M.	F			
32 M.H.	F			
2 P.D.	NC			
10 J.R.	NC			

SN Seronegative

NC No change in titre

F Fall in titre

R Rise in titre

Table 6.53 Changes in titre of IgG anti-MA up to 10 weeks from the onset of treatment in Sri Lankan patients with invasive carcinoma with known follow up histories

	TOTAL	RECURRENCE PATIENTS	TERMINAL CASES	WELL PATIENTS
Total	20	6	1	13
Rise in titre	7	2 (33%)		5 (38%)
Fall in titre	2	0		2 (15%)
Unchanging titre	8	1 (16%)	1	6 (46%)
Seronegatives	3	3 (50%)		

Table 6.54 Changes in titre of IgA anti-MA up to 10 weeks from the onset of treatment in Sri Lankan patients with invasive carcinoma with known follow up histories

	TOTAL	RECURRENCE PATIENTS	TERMINAL CASES	WELL PATIENTS
Total	20	6	1	13
Rise in titre	0	0		0
Fall in titre	7		1	6 (46%)
Unchanging titre	6	1		5 (38%)
Seronegative	7	5		2 (16%)

Table 6.55 Immunoglobulin (IgG and IgA) and membrane antibody (IgG and IgA) in 8 Sri Lankan well patients after 10 weeks from treatment - comparison with British well patients and British recurrence patients

A.

	TOTAL IgG	TOTAL IgA	IgG ANTI-MA	IgA ANTI-MA
Proportion of Sri Lankan well patients showing rise in level or titre	$\frac{3}{8}$ (38%)	$\frac{3}{8}$ (38%)	$\frac{4}{8}$ (50%)	$\frac{0}{8}$
Proportion of British well patients showing rise in level or titre	$\frac{5}{15}$ (33%)	$\frac{6}{15}$ (40%)	$\frac{5}{15}$ (33%)	$\frac{5}{15}$ (33%)

B.

	*↑IgG ANTI-MA ↑TOTAL IgA	↑IgG ANTI-MA ↑TOTAL IgG	↑TOT. IgA ↑TOT. IgG	↑IgG ANTI-MA ↑TOTAL IgG ↑TOTAL IgA
Sri Lankan well patients	$\frac{1}{8}$ (13%)	$\frac{2}{8}$ (25%)	$\frac{0}{8}$	$\frac{0}{8}$
British well patients	$\frac{2}{15}$ (13%)	$\frac{1}{15}$ (6%)	$\frac{4}{15}$ (26%)	$\frac{1}{15}$ (6%)
Sri Lankan and British well patients	$\frac{2}{23}$ (13%)	$\frac{3}{23}$ (13%)	$\frac{4}{23}$ (17%)	$\frac{1}{23}$ (4%)
British recurrence patients	$\frac{3}{5}$ (60%)	$\frac{4}{5}$ (80%)	$\frac{4}{5}$ (80%)	$\frac{3}{5}$ (60%)

*↑ Rise in

of treatment have remained well up till now. In Table 6.55A a comparison has been made of the changes in total IgG and total IgA and IgG anti-MA and IgA anti-MA observed after a period of 10 weeks in these patients and in British patients who have remained well. The changes are similar. Furthermore on grouping the Sri Lankan and British patients who have remained well together (row 3 of Table 6.55B), and comparing the combined results with those of British patients who developed recurrences, a rise intotal IgG and a rise in IgG anti-MA gives the best differentiation between patients who developed recurrences and those who remained well.

TOTAL IMMUNOGLOBULINS (IgG AND IgA) AND IgG AND IgA ANTI-BODIES TO ANTIGENS OF HSV-2 INFECTED CELLS IN THREE BRITISH PATIENTS WHO DIED FOLLOWING RECURRENCE OF CARCINOMA

Table 6.56 shows the levels of total IgG, total IgA, IgG anti-MA and IgA anti-MA antibody in three patients who died following recurrence of carcinoma. First serum samples from these patients had been collected at varying intervals of time after initial therapy, i.e. it was not possible to determine pre-treatment levels of immunoglobulin and titres of antibody.

Patient No. 1 appeared to be progressing well by clinical examination on 28/2/77. In her first serum sample which was collected 40 months after initial treat-

ment, she had levels of total IgG (186 iu/ml) and total IgA (179 iu/ml) which were higher than the GML of controls (132 iu/ml and 83 iu/ml respectively). A tumor recurrence was suspected on 25/7/77 and confirmed on 25/8/77. Between these two visits she showed a rise in level of total IgG. The patient died one year later.

Patient No. 2 had a stage III carcinoma and her first sample of serum was taken one month after start of treatment when levels of IgG and IgA above normal levels was detected (211 iu/ml and 157 iu/ml respectively). She showed a rise in level of IgG and titre of IgG anti-MA on 4/7/77 after which recurrence of carcinoma was noticed on 21/11/77. A pelvic clearance was done on 13/1/78 after which there was a fall in level of IgG noticed on 13/4/78. Total IgA levels rose after pelvic clearance and remained high. She also showed a rise in titre of IgG anti-MA on 3.4.78. The patient died one year after her last serum was collected.

Patient No. 3 had levels of IgA (79 iu/ml) within normal limits and showed no clinical signs of recurrence up to 17/10/77. She showed a rise in titre of IgG anti-MA between 21/2/77 and 17/10/77 and a progressive rise of total IgA from 21/2/77 to 16/1/78. A recurrence was suspected on 22/12/77. The patient died six months later on 11/6/78.

In all three patients an elevated total IgG level appeared to drop at the time recurrence of the carcinoma was developing, e.g. patient No. 1 on 25/7/77. patient No. 2 on 5/12/77

and patient No. 3 on 16/1/78.

Changes in level of IgA anti-MA did not relate to clinical findings.

PATIENT NAME & NO. DATE TREATMENT WAS WAS STARTED	DATE OF COLLECTING SERUM	SERUM NO. & TIME AFTER START OF TREATMENT	TOTAL IgG IN iu/ml	TOTAL IgA IN iu/ml	IgG ANTI-MA TITRE	IgA ANTI-MA TITRE	RECURRENCE SUSPECTED OR DETECTED
1. E.L. 10.10.73	28. 2.77	S_1 40 months	186	179	256	16	No. rec.
	25. 7.77	S_2 45 months	113	179	128	32	Rec. suspected Smear Pos.[4]
	25. 8.77	S_3 46 months	186	95	128	16	Recurrence confirmed
	26. 9.77	S_4 47 months	127	132	128	32	
	31.10.77	S_5 48 months	139	172	128	32	
Died	16. 8.78	58 months					
2. C.M. 1.12.76	30.12.76	S_1 1 month	211	157	256	8	
	4. 7.77	S_2 8 months	252	159	128	8	
	21.11.77	———	———	———	———	———	→ Recurrence
	5.12.77	S_3 13 months	184	128	128	8	
	13. 1.78	———	———	———	———	———	→ Pelvic clearance
	17. 1.78	S_4 14 months	184	149	64	8	
	3. 4.78	S_5 17 months	159	147	256	8	
Died	April '79	28 months					
3. E.N. April '76	15.11.76	S_1 7 months	not done	79	64	8	
	21. 2.77	S_2 10 months	not done	63	64	4	
	17.10.77	S_3 14 months	123	70	128	8	
	22.12.77	———	———	———	———	———	→ Recurrence suspected
	16. 1.78	S_4 22 months	109	81	128	4	
Died	11. 6.78	22 months					

Geometric mean levels of controls

IgG	131 iu/ml
IgA	83 iu.ml

Table 6.56 Changes in level of total IgG and IgA and IgG anti-MA and IgA anti-MA in 3 British patients with invasive carcinoma who died of recurrence.

RESULTS. PART VIII.

A. PREVALENCE OF ANTIBODIES TO HERPES SIMPLEX, RUBELLA AND MEASLES IN BRITISH, SRI LANKAN AND MALAWIAN SUBJECTS.

B. CHANGES IN TITRE OF ANTIBODIES TO 3 VIRUSES FOLLOWING TREATMENT OF PATIENTS WITH INVASIVE CARCINOMA BY RADIOTHERAPY.

PART VIII

PREVALENCE OF HERPES, RUBELLA AND MEASLES VIRUSES AMONG POST-PUBERTAL FEMALES IN BRITIAN, MALAWI AND SRI LANKA

Only the serum of controls was used in estimating the prevalence of herpes antibodies. However, the sera of both patients and controls was used in estimating the prevalence of rubella and measles antibodies.

A high prevalence of herpes and measles antibodies was found in all three populations, and of rubella in Britain and Malawi. The prevalence of rubella antibody among the Sri Lankan subjects, however, was significantly lower (X^2, $P<.01$) than among the British and Malawian subjects. (Table 6.57).

CHANGES IN TITRE OF IgG ANTI-MA AND RUBELLA AND MEASLES HAEMAGGLUTINATION INHIBITION (HI) ANTIBODIES IN PATIENTS WITH INVASIVE CARCINOMA FOLLOWING RADIOTHERAPY

Many British and Sri Lankan patients with invasive carcinoma showed a rise in titre of IgG anti-MA immediately following radiotherapy. Therefore rubella and measles HI titres were tested in these same sera in order to ascertain if the rise in antibody was herpes specific or part of a more general phenomenon due to the effects of radiotherapy.

Ten of 47 (21%) British and Sri Lankan patients with cervical carcinoma showed 4-fold rises in titre of IgG anti-MA compared with 3 of 41 (7%) who showed rises in rubella HI titres (Table 6.58). This difference was not

significant ($P < .05$). The difference however was highly significant on including in the comparison two-fold rises in titre as well. Twenty-seven of 47 (57%) of British and Sri Lankan patients then showed rises in titre of IgG anti-MA in comparison with 9 of 41 (22%) who showed rises in titre of measles HI antibody and 5 of 33 (15%) who showed rises in titre of rubella HI antibody ($P < .0005$) (Table 6.59).

On testing sucrose gradient fractions of the serum of one of the patients who showed a four-fold rise of both measles HI antibody and rubella HI antibody, antibody to measles antigen was detected only in the IgG fractions.

Table 6.57 Prevalence of measles, rubella and herpes simplex antibody in British, Sri Lankan and Malawian subjects.

	BRITAIN	SRI LANKA	MALAWI
Measles	$\frac{43}{45}$ (96%)	$\frac{37}{41}$ (90%)	$\frac{28}{28}$ (100%)
Rubella	$\frac{45}{45}$ (100%)	$\frac{32}{41}$ (78%)	$\frac{29}{29}$ (100%)
Herpes IgG anti-MA	$\frac{61}{66}$ (92%)	$\frac{17}{17}$ (100%)	$\frac{18}{18}$ (100%)

Table 6.58 Proportion of British and Sri Lankan patients with invasive carcinoma showing a 4-fold rise in herpes IgG anti-MA antibody and rubella and measles HI antibody up to 20 weeks from the onset of radiotherapy.

	HERPES IgG ANTI-MA	MEASLES HI	RUBELLA HI
British Inv. Ca. patients showing 4-fold rises	$\frac{3}{23}$	$\frac{3}{23}$	$\frac{1}{23}$
Sri Lankan Inv. Ca. patients showing 4-fold rises	$\frac{7}{24}$	$\frac{4}{19}$	$\frac{2}{10}$
Total British and Sri Lankan	$\frac{10}{47}$ (21%)	$\frac{3}{41}$ (7%)	$\frac{3}{33}$ (9%)

$P > .05$ (X^2 - 2 tailed)

$P < .05$ (X^2 - 1 tailed)

Table 6.59 Proportion of British and Sri Lankan patients with invasive carcinoma showing a 2-fold and 4-fold rise in herpes IgG anti-MA antibody and rubella and measles HI antibody up to 20 weeks from the onset of radiotherapy.

	HERPES IgG ANTI-MA	MEASLES HI	RUBELLA HI
British Inv. Ca. patients showing 2-fold rises and 4-fold rises	$\frac{14}{23}$	$\frac{5}{22}$	$\frac{3}{23}$
Sri Lankan Inv. Ca. patients showing 2-fold rises and 4-fold rises	$\frac{13}{24}$	$\frac{4}{19}$	$\frac{2}{10}$
Total British and Sri Lankan	$\frac{27}{47}$ (57%)	$\frac{9}{41}$ (22%)	$\frac{5}{33}$ (15%)

$P < .0005$ (X^2 - 2 tailed)

RESULTS. PART IX.

LEVELS OF CARCINOEMBRYONIC ANTIGEN IN THE SERA OF BRITISH PATIENTS WITH INVASIVE CARCINOMA AND OF MATCHED CONTROLS.

RESULTS PART IX

LEVELS OF CARCINOEMBRYONIC ANTIGEN IN THE SERA OF BRITISH PATIENTS WITH INVASIVE CARCINOMA AND OF MATCHED CONTROLS.

Levels of carcinoembryonic antigen (CEA) were assayed in pre-treatment serum samples of 29 British patients with invasive carcinoma, and of 18 matched controls. In addition levels were assayed in serial post-treatment samples of 27 of these patients. Patients had higher pre-treatment levels (mean, 13.5 μg/litre) than controls (8 μg/litre), but this difference was not significant.

The purpose of estimating levels in serial samples was to observe if rising levels were an index of tumor recurrences. However it was found that similar proportions of patients who still remain well (63%) showed rising levels as patients who developed recurrences (57%) (Table 6.60).

Table 6.60 Changing levels of CEA in serial samples of serum of 27 British patients with invasive carcinoma.

	TOTAL	RECURRENCE PATIENTS	WELL PATIENTS
Total	23	7	16
Rise in level	14	4 (57%)	10 (63%)
Fall in level	8	3 (43%)	5 (31%)
No change in level	1		1 (6%)

Chapter 7

DISCUSSION & CONCLUSIONS.

SCREENING FOR CERVICAL CANCER IN THE U.K.

The mortality trends in countries which have introduced widespread cervical screening leave no doubt that it saves lives (Cervical Cancer Screening Program, 1976). A recent study by Clarke and Anderson (1979) in Ontario, Canada has provided convincing evidence of the efficacy of Papapicolaou smear screening in reducing the incidence of invasive cervical cancer. In Britain, the age at which screening should begin still remains debatable (Lancet, 1978). However, the age at which screening is begun is obviously a variable which every country must decide for itself depending on the epidemiological features and trends of the disease, and the resources available.

Trends in mortality from cervical carcinoma (England and Wales, 1957-1975)

In a cohort analysis according to year of birth (Fig. 7.1) mortality was generally decreasing in cohorts born up to the turn of the century, after which there was an increase reaching a peak in those born around 1921. This peak is attributed to a period of disturbed sexual relationships during the 1939-1945 war when this cohort of women reached their young adult life (Hill and Adelstein, 1967). There was a subsequent decline in mortality lasting approximately fifteen years after which there has been an increase in mortality affecting young women born in the 1940's or later. This difference is believed to be related to an increasing exposure to sexually transmitted infection, although not necessarily causally (Beral, 1974), early onset of coitus, increasing use of the pill and

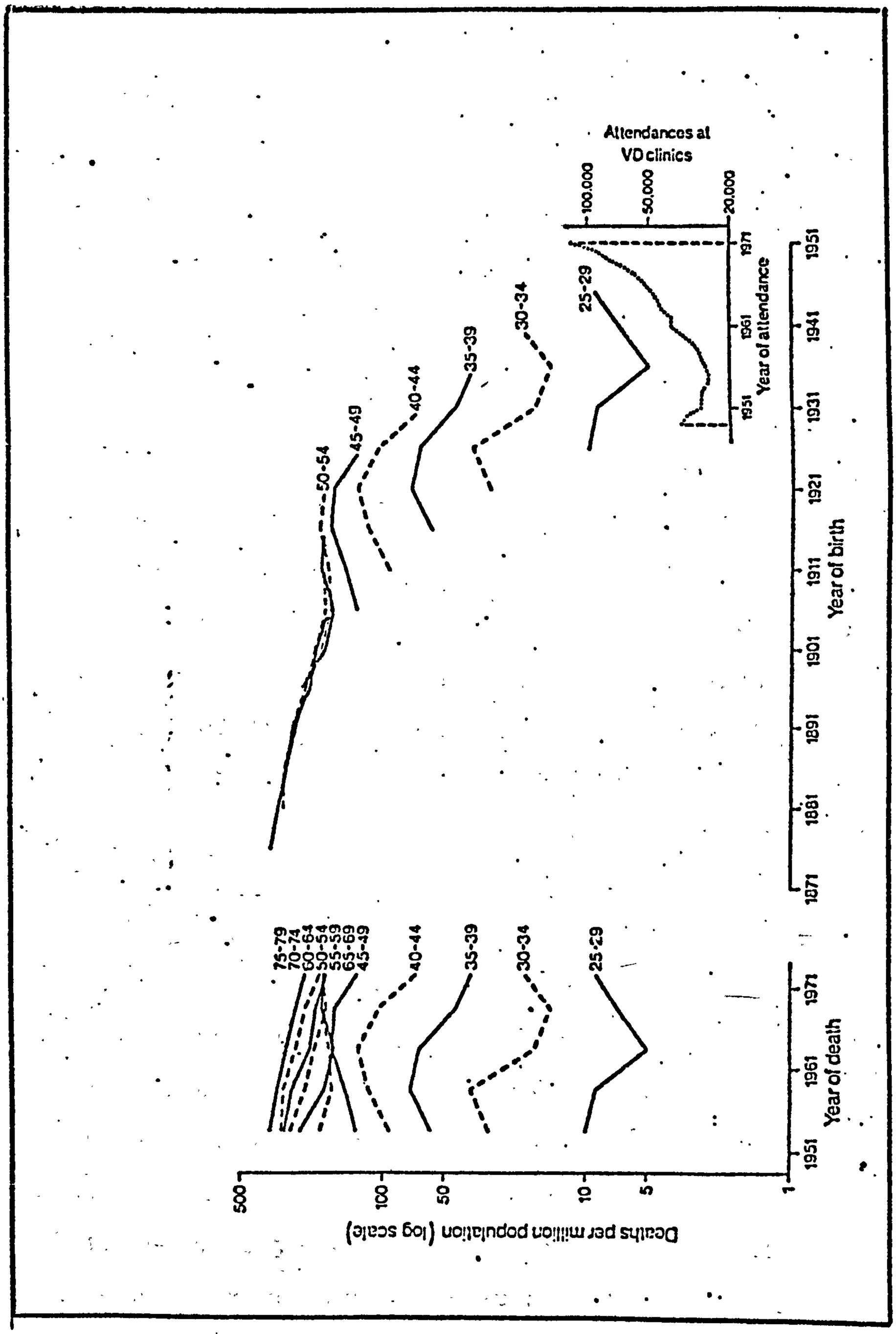
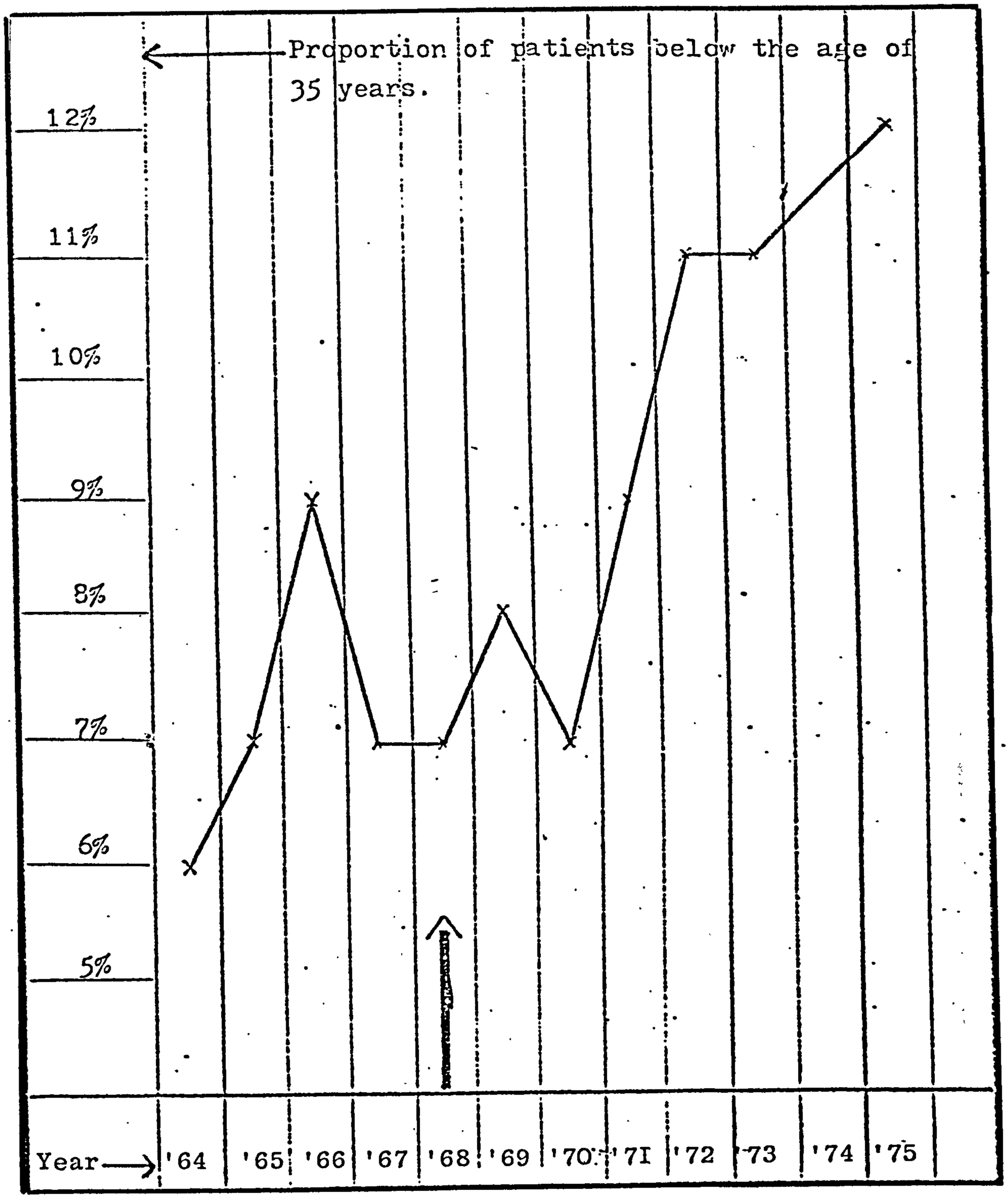


Fig. 7.1 Deaths by age from carcinoma of the cervix in England and Wales, according to year of death, 1951-1971, and year of birth. Also attendances of women at hospital VD clinics, 1949-1971.
(OPCS, 1978)

decreasing use of barrier contraception (Lancet, 1978). In fact the number of deaths among women between the ages of twenty-five and thirty-four have more than doubled between 1966 and 1976 from twenty-nine to sixty-nine (Lancet, 1978). This has caused the present screening policy of the Department of Health and Social Security (DHSS) to be questioned and there have been calls to reduce the age at which routine screening should begin (Lancet, 1978). At present the recommended age for routine screening is thirty-five years. One of the arguments that has been brought forward against altering the present screening policy is that although there has been a rise in mortality among younger women, the deaths in this age group still account for only a small number of the total deaths from cervical carcinoma, i.e. the sixty-nine deaths in 1976 were only 3% of the total number of deaths from cervical carcinoma for that year. Thus it has been argued (Spriggs and Husein, 1978) that many millions of smears would have to be screened in order to save one life. However, in the study reported in this thesis it was found that the incidence of cervical anaplasia was high in this age group. Thirty-nine of one hundred sixty-six (23%) patients with cervical anaplasia were aged thirty years or younger. Of these, twenty-three had dysplasia, eleven had carcinoma *in situ* and five had invasive carcinoma, i.e. sixteen (44%) of a total of one hundred fourteen patients requiring immediate treatment (patients with carcinoma *in situ* and invasive carcinoma) were thirty years or younger. Since it was felt that age specific incidence or morbidity information rather than mortality information was a better criterion of assessing the problem



Start of screening programme in England and Wales.

Figure 7.2 Proportion of women below the age of 35 years among all women presenting with cervical carcinoma - England and Wales (1964 - 1975).
Based on figures in the Hospital In-Patient Enquiry.

of cervical carcinoma in the younger age group an attempt was made while writing this thesis to obtain these figures for England and Wales. It was discovered that comprehensive information on age specific incidence is not readily available. Therefore an approximate estimate was made from the Hospital In-Patient Enquiry (HIPE) published by the Office of Popula-tion Censuses and Surveys (OPCS) which contains statistical analysis of one in ten discharges and deaths occurring in all hospitals in England and Wales. Information was available only for patients with invasive carcinoma, and not for patients with dysplasia and carcinoma *in situ*, and these figures confirm the findings in this study. In 1964, 6.1% of all patients admitted with invasive carcinoma were below the age of thirty-five years. By 1975, this proportion had doubled to 12% (Fig. 7.2). The estimated number of patients under the age of thirty-five admitted with invasive carcinoma in 1964 was seven hundred twenty-eight. By 1976 this number had risen to one thousand four hundred fifty-nine. In this study, the ratio of patients below thirty years with invasive carcinoma, to those with dysplasia and carcinoma *in situ* was one to seven. Thus the figures quoted above on the incidence of cervical carcinoma among the young women probably represents the tip of the iceberg as regards the incidence of all forms of cervical anaplasia (dysplasia, carcinoma *in situ* and invasive carcinoma) in this age group.

Screening for cervical cancer in post-menopausal women

Thirty-six of seventy patients with invasive carcinoma in this study were aged fifty to seventy years and of the

twelve patients who died as a result of recurrences, seven were also in this age group. The fact that over 50% of all women with cervical carcinoma in this study were post-menopausal women implies that the screening programme is not sufficiently made available to and promoted in this age group.

Implications of the social class analysis of British patients with cervical anaplasia

A major proportion of patients with dysplasia (75%), carcinoma in situ (73%) and invasive carcinoma (66%) were in social classes I - III. While this may be a reflection of the social class distribution of the population served by St. Thomas', it is still a finding contrary to the accepted belief that cervical carcinoma is associated with lower social class. Thus it may reflect a change in the epidemiology of the disease which has been brought about by the change in sexual mores and habits of the people. Thirty per cent of patients with invasive carcinoma were in the lower social classes (IV and V) in contrast with 8% of patients with dysplasia and 20% of patients with carcinoma in situ. This probably reflects the fact that women in the lower socio-economic groups present less frequently for cervical smears and are therefore seen when they develop symptoms caused by invasive carcinoma. This is a commonly encountered problem in cytology screening.

The efficiency of the screening programme.

Figure 7.3 shows the mortality rates for cervical carcinoma per one hundred thousand of the population in England and

Wales between the years 1964 and 1976. In Figure 7.4, an approximate estimate of incidence has been made up to 1975 from information obtained from the HIPE. The estimated number of total admissions with invasive carcinoma have been related to the estimated mid-year home population in England and Wales for that year, according to sex and age. It is seen that the screening programme which began in 1968 has not had any impact on either.

The success of a screening programme depends on such factors as:

a) The population of women at risk who are screened

b) The taking of smears by the correct method

c) The correct reading of smears

d) The efficient follow up of patients with abnormal smears.

While the proportion at risk who are being screened in Britain has not been estimated, this proportion has been high in areas of the world where screening programmes have achieved good results. In British Columbia, where all sexually active women above the age of twenty were screened, a 33% reduction in incidence rate was achieved in seventeen years (Worth, 1973). In Toledo, USA over a period of nineteen years the proportion being screened was stepped up gradually from 15-90% of those at risk. A 66% reduction in the incidence rate was achieved (Kim et al, 1978). Similarly in Louisville, Kentucky where screening began in 1956, 94% of the population at risk were being screened by 1967. A 57% decrease in incidence rates and a 51% decrease in mortality was observed from 1955-1973 (Christopherson, 1976).

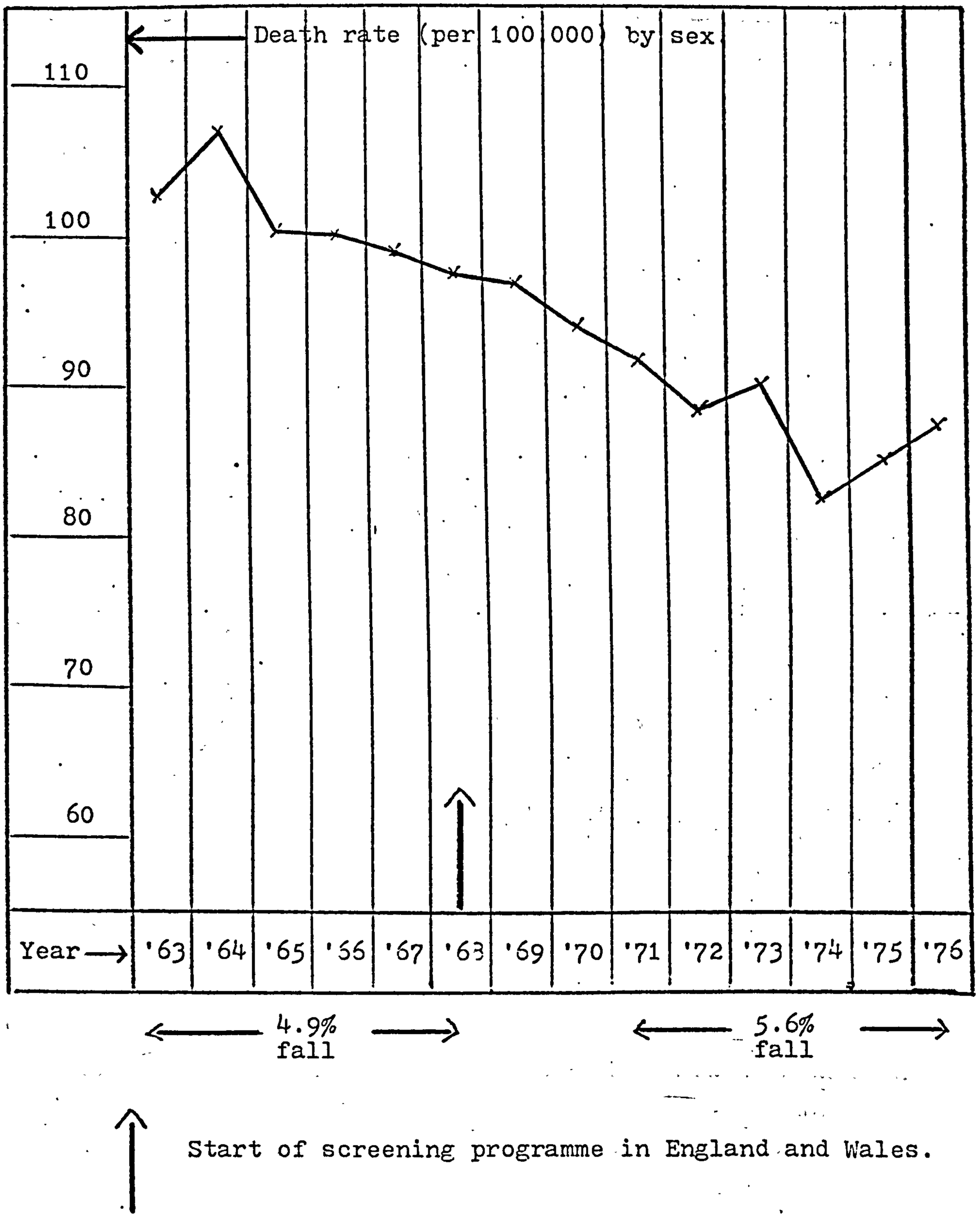
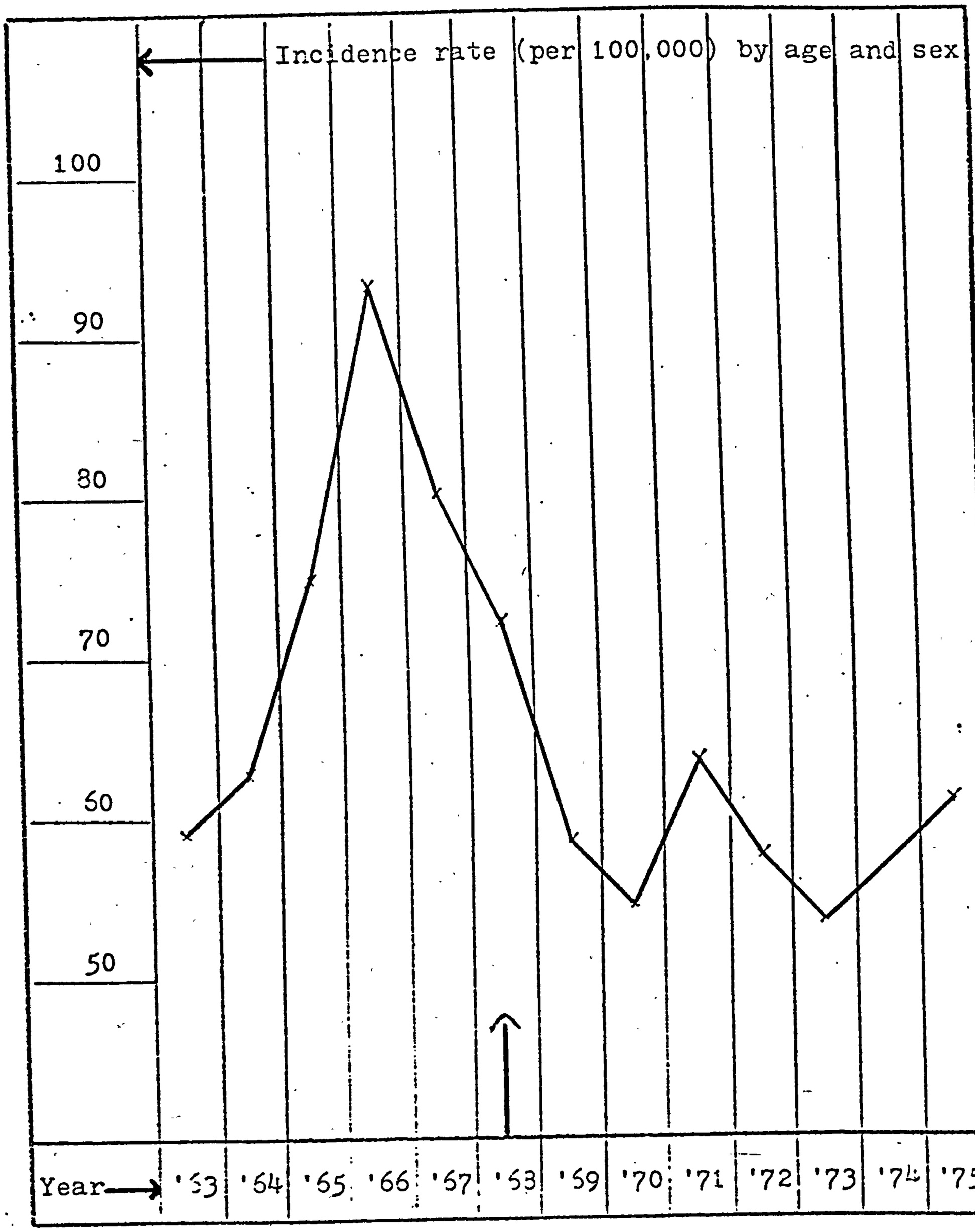


Figure 7.3 Death rates from cervical carcinoma - England and Wales (1963 - 1976). From mortality statistics by cause, Office of the Population Censuses and Surveys.



Start of screening programme in England and Wales.

Figure 7.4 Incidence rates for cervical carcinoma - England and Wales (1963 - 1975) based on figures in the Hospital In-Patient Enquiry.

In contrast, in Auckland where approximately only 20% of the women at risk were being screened, the incidence and mortality rates which were already falling prior to the campaign did not show an accellerated response (Green, 1970).

There are basically two criteria by which the cost benefit of widespread cervical screening and thereby the detection of early cervical lesions may be assessed: a) the benefit to the patient and b) the benefit to the Health Services. The treatment of early invasive carcinoma involves extensive operative or radiotherapeutic procedures which are often debilitating and which always castrate. Thus, though the cure rates in early cervical carcinoma are good, the price is high. Compared with this, the benefit to the patient of a comparatively simple curative procedure applied to pre-invasive lesions which involves the removal of selected areas of abnormal cervical epithelium, are only too obvious. In terms of the expenses incurred by the Health Services in treating the more advanced lesions, it was estimated in the U.S.A. that for every dollar spent, nine were saved (Christopherson, 1976).

Ethnic origin of patients with cervical anaplasia

Studies which showed varying incidence of cervical carcinoma between women sharing the same environment but belonging to different ethnic groups were described previously in the Introduction (page 79). In the studies reported in this thesis 21% of British patients below the age of thirty years with cervical anaplasia were West Indian in origin. Although their socio-economic distribution was similar to that of the Caucasians, a higher proportion of them had had previous

venereal infection or had a history of previous spontaneous or therapeutic abortion. In addition a smaller proportion of West Indians in this age group were married. Although numbers are small, they may imply that different ethnic groups in Britain carry varying degrees of risk of developing cervical carcinoma. Ethnic group associated incidence has not been adequately established in the U.K. It is of interest that in a study conducted in the genito-urinary department of St. Thomas' Hospital, it was noticed that dysplasia was nearly twice as common among Negroids with genital herpes than among Caucasians with genital herpes (Carrol, 1977).

AGE OF DEVELOPING CERVICAL CARCINOMA IN SRI LANKAN, MALAWIAN AND SUDANESE WOMEN

In contrast with British women with invasive cervical carcinoma, only a low proportion (6%) of Sri Lankan women presented in stage one of the disease. Despite this the age group showing the maximum incidence of malignancy was younger (forty-one to sixty) among Sri Lankan than British women (fifty-one to seventy). The age group showing maximum incidence of the tumor was even younger among Malawian and Sudanese women (thirty-one to fifty). Furthermore, like Sri Lankan patients, Sudanese and Malawian patients presented in the later stages (Stages II and III) of the disease. It is apparent from the results on age incidence and on considering the stage of disease that they presented in, that the tumor develops well within the child-bearing age in a high proportion of Sri Lankan, Malawian and Sudanese women. Thus there is seen an obvious need to screen these women for cervical cancer at least at the antenatal clinics, more so because cervical carcinoma is one of the commonest female tumors in all these three countries.

In the historical survey seven criteria were layed down which if fulfilled would provide evidence that HSV-2 was aetiologically related to carcinoma of the cervix (page 24). Clearly this study could not contribute further evidence to criterion number five and six. However it contributes towards the other five criteria, and this is discussed below.

CRITERION NUMBER 1

THE EPIDEMIOLOGICAL CHARACTERISTICS OF WOMEN WITH CERVICAL CARCINOMA AND GENITAL HERPES SHOULD BE SIMILAR.

As many studies have demonstrated before (page 6), in this study too early initiation of sexual intercourse was a noticeable feature among patients from all four countries, e.g.

Sri Lankan patients.

The date of marriage is usually the date of first coitus among Sri Lankan women. In this study the average age of patients with invasive carcinoma was seventeen years in contrast with controls who had married at an average age of twenty-two years.

Malawian patients.

Approximately 50% of Malawian patients and controls had married before the age of eighteen years. One patient had married before menarche at the age of eleven years and two at the ages of fourteen and fifteen. Such early initiation of young girls into marital relationships shortly after menarche is a cultural feature among many African communities such as in Uganda, Zambia and Malawi(S.Palaniappan , Personal communication).

Sudanese patients

Although the date of marriage of patients in the study could not be obtained (see Materials and Methods page 112), independent information obtained about marriage customs among Sudanese (M. Kambal and M.O.A. Malik, Personal communication) revealed that marriages are often arranged for Sudanese females between the ages of fifteen and nineteen years.

British patients

Unfortunately the gynaecologists at St. Thomas' were not willing that their patients should be questioned about the age of initiation of coitus. The average age of marriage of British patients was twenty to twenty-one years. Table 7.1 was compiled by Chamberlain and Dewhurst (1977) from two studies conducted by Michael Schofield. One of these studies (Schofield, 1965) aimed to measure the sexual experience of British males and females between the ages of fifteen and nineteen. His research was conducted on random samples drawn from seven areas in London which were representative of all socio-economic groups. He found that 17% of females had experienced sexual intercourse by the age of eighteen years. From another study by Schofield, Chamberlain and Dewhurst quote 70% of British females as being sexually experienced before marriage.

Table 7.1 Sexual experience of young adults in Britain by age (from Chamberlain and Dewhurst, 1977), based on two studies by Michael Schofield).

	PROPORTION SEXUALLY EXPERIENCED	
	MALE	FEMALE
By 16 years	14%	5%
By 18 years	34%	17%
By 21 years	75%	71%
By 25 years	92%	94%
Before marriage		70%

By extrapolating from this indirect evidence it is likely that many of the British patients in this study who had married between the ages of twenty and twenty-one years had had sexual intercourse in early adolescence. Thus how does the herpes hypothesis fit in with the constant epidemiological finding that women with cervical anaplasia begin sexual activity at an early age? Two possibilities have already been briefly mentioned in the historical survey (pages 25 & 26).

Firstly Coppleson and Reid (1967) suggested that the metaplastic adolescent-cervical epithelium is more likely to be transformed by a sexually transmitted agent into cells possessing neoplastic potential, than the non-metaplastic epithelium in later life. Is HSV-2 infection then acquired in early adolescence, thus fulfilling the triad of events represented in Figure 7.5?

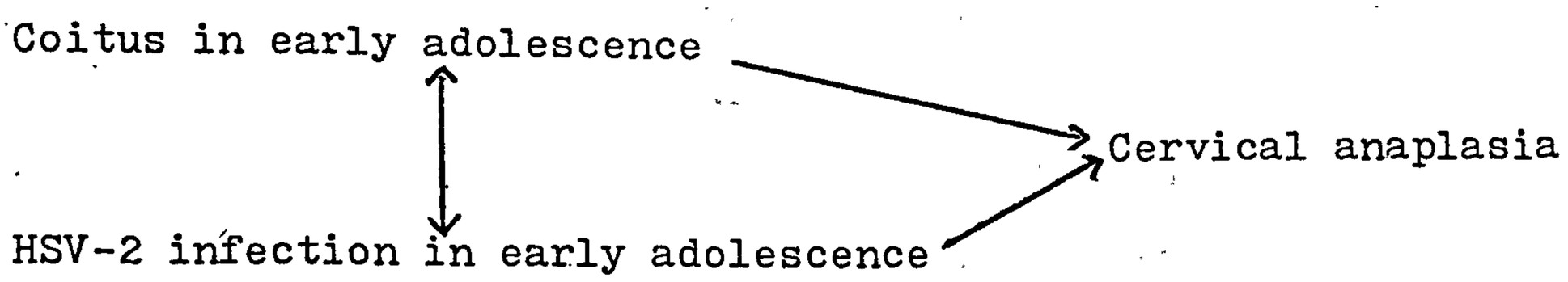
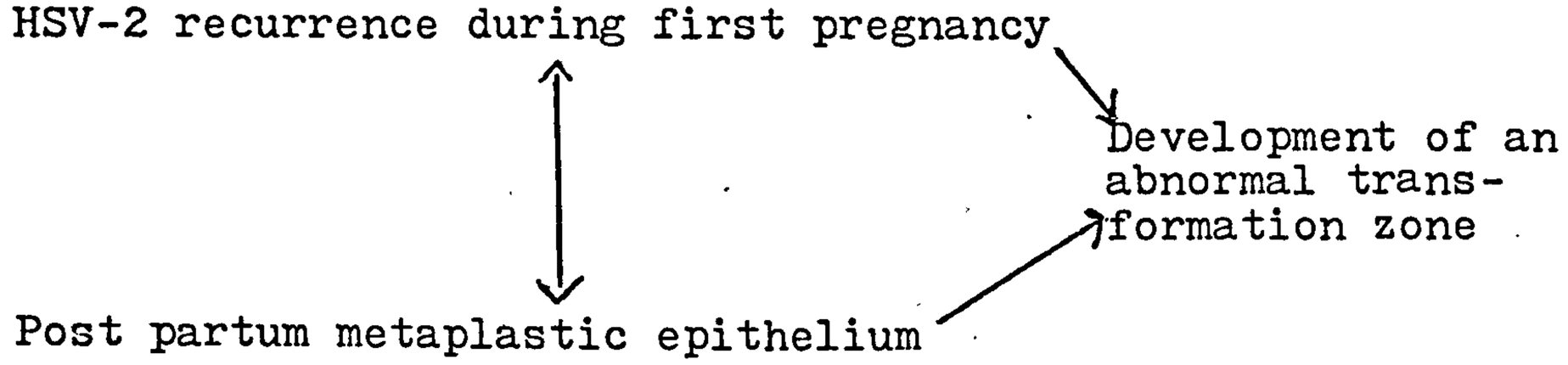


Figure 7.5 A suggested triad of events leading to cervical anaplasia if the herpes hypothesis is to fit the observed epidemiological features of cervical carcinoma.

The average age of twenty patients with genital herpes in this study was 23.5, the median 21.5 and the range 18-39 years. In the study conducted by Naib et al (1969) the average age was 20 years. In a study conducted at the genito-urinary department at St. Thomas', 32% of three hundred thirteen patients with genital herpes presented between the ages of thirteen years and nineteen years (Carrol, 1977). Over 50%

of cases of genital herpes seen recently at the venereal disease clinic, of the General Hospital in Birmingham, has been in patients under the age of twenty-one years (Skinner et al, 1978). This provides evidence that a reasonable proportion of HSV-2 genital infection does in fact occur in early adolescence. It also leads to the possibility that the selective factor which operates is the age at which HSV-2 genital infection occurs, i.e. it could be that of patients of all ages who acquire HSV-2 genital infection only those acquiring it during early adolescence progress to develop cervical anaplasia. This possibility has so far not been explored adequately but if established would explain the discrepancy between the calculated risk of a woman developing genital herpes which is 0-80% (average 20%) and the calculated risk of a woman developing cervical anaplasia which is 1-2% (Nahmias et al, 1978). An explanation for this discrepancy is indeed essential if a causal relationship in cervical anaplasia was attributed to the virus. What evidence then may confirm or strengthen this possibility? If it could be shown by coloscopy that of all patients suffering from HSV-2 genital herpes only those infected before the age of nineteen years - develop colposcopically abnormal transformation zones, the association between early age of coitus, HSV-2 infection and cervical anaplasia would be strengthened. However, two factors have to be borne in mind: a) that the cervical epithelium also undergoes metaplasia following first pregnancy and b) that it has been observed that recurrent attacks of genital herpes are more common in pregnancy (Naib, 1966; Yen et al, 1965).

Thus colposcopic examination of the cervices of women with a previous history of genital herpes or those with HSV-2 specific antibodies after first pregnancy would yield evidence for the second possible triad of events.



Since both metaplastic periods in the epithelium are transient this hypothesis takes into account the selective factors of exact timing in acquiring a genital herpes infection, i.e. infection and metaplasia must be concurrent.

The second suggestion attempting to link early initiation of coitus with the herpes hypothesis (Rawls et al, 1976) is that women who begin sexual activity at an early age also have multiple sexual partners. This is possibly true of more sexually liberated Western societies but is not strictly true of more conservative Asian communities such as those in India, and Sri Lanka or such Muslim communities as in The Sudan, where the tumor is one of the commonest in women. However, in this study, a high proportion of patients with invasive carcinoma from both Sri Lanka and Sudan possessed IgA anti-MA to HSV-2 infected cells (78% and 85% respectively) and HSV-2 specific antibodies by ELISA (65% and 52% respectively). Thus while the investigation into the social habits of these two study groups is not in keeping with the suggestion of Rawls and his colleagues, an alternative explanation must be considered

which may possibly operate in these more conservative societies. This relates to recent evidence focusing on the importance of the high risk male. Kessler (1977) identified men who were married to women with confirmed cervical cancer at a time prior to the date of cancer diagnosis, and subsequently traced one thousand eight-seven other wives of these men. The incidence of abnormal cervical cytology in these one thousand eighty-seven women was 14% and this contrasts with an incidence of 8% in six hundred fifty-nine control wives. Twenty-nine "marital clusters" were identified in which two women married to the same man had developed cervical neoplasia. The expected number of such "marital clusters" was 11.6. Centifanto et al (1972) isolated HSV-2 from the urethral discharge of 15% of one hundred ninety male patients seen at a urology clinic none of whom had a known history of genital herpes. Similar isolation rates have been reported by others (Goodwin et al, 1970; Jeansson and Molin, 1971). In both Sri Lankan and Sudanese societies, pre-marital and extra-marital sexual activity, although generally uncommon, is a more likely feature among the males in the community with a restricted population of willing females. Thus in these communities HSV-2 could well have been transmitted to a cloistered sexually, inexperienced, monogamous young female by an older more experienced male whom she married. Unfortunately, well designed epidemiological studies in such communities are sadly lacking, although these communities provide interesting epidemiological groups in whom both the role of the male as well as the mechanism of acquiring HSV-2 genital infection at an early age may be studied.

CRITERION NUMBER 2

WOMEN WITH CERVICAL CARCINOMA WOULD BE EXPECTED TO HAVE A HIGHER FREQUENCY OR HIGHER TITRES OF ANTIBODY TO VIRUS SPECIFIED ANTIGENS AND ASSAYS OF CELL MEDIATED IMMUNITY SHOULD SHOW A POSITIVE OR NEGATIVE CORRELATION

The antibodies to three types of viral antigens were examined in this study:

A. The membrane antigens on the surface of HSV-2 infected cells.
B. The virus capsid antigens in the cytoplasm and nucleus of HSV-2 infected cells.
C. Antigens which are type-specific to HSV-1 and HSV-2.

A. & B. ANTIBODIES TO MEMBRANE ANTIGENS AND VIRUS CAPSID ANTIGENS OF HSV-2 INFECTED CELLS.

Sir MacFarlane Burnet coined the term immune surveillance when he elaborated a theory expounded by Lewis Thomas that the immune system is the basic natural machanism against neoplasia, and that cancer cells differ from normal cells and are recognised by the immune system as being foreign. The tumor antigens that the immune system can most effectively respond to and therefore reject are those located on the cell membrane rather than in the cell interior. In the Introduction of this thesis, studies were quoted which had demonstrated HSV related antigens on the cell membranes of squamous carcinoma cells, on cells transformed by HSV, and cells infected by HSV. Also in the Introduction, new non-virion antigens, Fc receptors, lectin receptors, unmasked fetal antigens and histocompatibility antigens were described as possible new antigens which could be detected in HSV infected cells. In the case

of adenoviruses, a non-virion antigen has been demonstrated which is common to cells undergoing lytic infection by the virus as well as on tumors induced by the virus. Thus, the basis for employing HSV-2 infected cell membranes as the antibody trapping test system in this study was that the antigens of these membranes would at least partly represent the antigens on a cervical carcinoma cell, if the virus was aetiologically related to the tumor.

In the studies reported in this thesis the results of the IgG and IgA membrane fluorescent assays showed that whereas both the frequency of IgG anti-MA and the titres were similar among patients with cervical anaplasia and controls, both the frequency of IgA anti-MA and their titres differed. A higher proportion of British and Sri Lankan patients with cervical anaplasia possessed IgA anti-MA to HSV-2 infected cells and in significantly higher titres than controls and all other patients with malignancies except patients with squamous carcinomas at other sites of the body. All patients with genital herpes had persisitent IgA anti-MA and their titres were similar to those seen in patients with cervical anaplasia and with squamous carcinoma. IgA anti-MA persisted in a high proportion of British patients with invasive carcinoma who had been treated one to twelve years previously. The titres of IgA anti-MA in these patients were similar to those observed among untreated patients with cervical anaplasia, squamous carcinoma and patients who had had genital herpes.

As in studies reported by El Falaky and Vestergaard (1977), a similar proportion of patients with cervical anaplasia and controls possessed IgA anti-VCA antibodies and in similar titres. Titres of IgA anti-MA increased with increasing stage of disease.

The questions which arise from these results are as follows:

1. Against which antigens on the surface of the HSV-2 infected cell are these IgA anti-MA antibodies directed?
2. Why is there a high prevalence and a high titre of IgA anti-MA in patients with squamous carcinoma?
3. Why do these antibodies persist in high titre in patients with invasive carcinoma who had been previously treated?
4. How do these results relate to the hypothesis that HSV-2 causes cervical carcinoma?

Antigens against which the IgA anti-MA antibodies could be directed.

Current experimental work where attempts have been made to characterise the glycoproteins of HSV infected cells are relevant to the above questions.

Vestergaard et al (1977a), Vestergaard and Grauballe (1975), and Norrild and Vestergaard (1977) have by immunoelectrophoretic studies characterised some antigens on the cell membranes of HSV-1 and HSV-2 infected cells.

Ag 8 and Ag 11 are two major HSV type common membrane bound glycoproteins each composed of several polypeptides of which at least one is glycoselated. Monospecific antisera raised against these glycoproteins neutralised both HSV-1 and HSV-2 (Table 7.2). They were shown to contain polypeptides belonging to the high molecular weight region of the glycoselated polypeptides found in purified virions which were designated 7/8 by Spear and Roizman (1972), and 2/3 by Powell and Watson

(1975). Recent work suggests that both Ag-8 and Ag 11 from HSV-1 infected cells contain HSV-1 type specific determinants in addition to type common determinants but Ag-8 and Ag-11 from HSV-2 infected cells contain only type common determinants (Vestergaard and Grauballe, 1977; Vestergaard, 1979). Vestergaard (1979) using crossed immunoelectrophoresis with intermediate gel, estimated semiquantitatively the antibody against eight herpes simplex virus proteins in one hundred human sera. These sera possessed higher antibody titres to Ag-8 and Ag-11 than to other proteins. There was also a high degree of correlation between neutralising antibody titre and titre of antibody to Ag-8 and Ag-11. It is deduced therfore that most serological cross reactivity between HSV-1 and HSV-2 in human sera is caused by antibody to Ag-8 and Ag-11.

Ag-4, and to a smaller extent Ag-9 contain HSV-2 type specific determinants but also some type common determinants. Thus monospecific sera raised against these glycoproteins neutralise not only HSV-2 but to a smaller degree HSV-1 as well. Antibody against Ag-4 and Ag-9 were present in the one hundred human sera referred to above. The major part of antibodies directed against these two antigens are believed to represent HSV-2 specific antibodies. Ag-1 is totally HSV-2 type specific. However it does not appear to play a role in the human immune response (Vestergaard, Personal communication).

Ag-3 is a water soluble non-glycoselated type common antigen. Antibodies to Ag-3 were detected in human sera but in smaller quantities than antibodies to Ag-8 or Ag-11. The true nature of Ag-3 is not known. It might represent a

Table 7.2 Immunological data of HSV antigens extracted from infected cell membranes by non-ionic detergents (Adapted from Vestergaard et al, 1977).

ANTIGEN	VIRUS, ANTIGEN IS PRESENT	SOLUBILITY IN WATER	MEMBRANE BOUND	VIRUS, NEUTR'D BY MONOSPECIFIC SERUM TO	SIMILAR ANTIGENS DESCRIBED BY OTHER WORKERS
8	HSV-1 & HSV-2	-	+	HSV-1 & HSV-2	7/3 Spears & Roizman, 1972 and 2/3 of Powell & Watson, 1975
11	HSV-1 & HSV-2	-	+	HSV-1 & HSV-2	
3	HSV-1 & HSV-2	+	-	Weak neutr'n. of HSV-1 and HSV-2	
1	HSV-2	+	-	Does not neutralise either.	
9	HSV-2	-	+	Greater neutralizing potency against HSV-2 than HSV-1	? VP 119 Courtney
4	HSV-2	-	+		
6	HSV-1	-	+	Neutralises HSV-1	? VP 123 Courtney & Powell 1975

Table 7.3 Proportion of patients with different malignancies who possessed antibody to NVA-TAA - results of 2 studies.

DIAGNOSIS	NUMBER REACTING IN CF TEST TO NVA-TAA	REFERENCE AND TEST
Carcinoma of the larynx	36/38 (95%) *	Complement fixation test
Controls for CA of the larynx	2/36 (5%) *	
Squamous carcinoma	48/57 (84%)	Hollinshead & Tarro (1973)
Cases cured of sq. carcinoma	20/28 (71%)	
Carcinoma of the cervix	21/24 (87%)	
Non-squamous carcinomas	2/24 (8%)	
Sq. cancer of head and neck other than larynx	15/24 (62%)	
Carcinoma of the cervix	13/16 (81%)	Microcomplement fication test. Notter and Docherty (1976)
Squamous cell cancer of head, neck and vulva	6/8 (75%)	
Non-squamous carcinoma	3/11 (27%)	

major capsid antigen (Vestergaard, 1979).

Ag-6 is an HSV-1 type specific virion glycoprotein. Monospecific antibodies against Ag-6 neutralise HSV-1 only. Antibody to Ag-6 was detected in human sera. Together with the HSV-1 specific determinants on Ag-8 and Ag-11, Ag-6 is credited with eliciting HSV-1 specific antibody in human sera.

Vestergaard et al (1977a) have suggested, that Ag-6 of molecular weight 131,000 is identical to the major virion envelope glycoprotein reported by Spear and Roizman (1972) and Courtney and Powell (1975).

Courtney and Powell termed this major virion envelope glycoprotein of HSV-1 infected cells, VP 123. In a later study (presented at the herpes workshop, Cambridge, England in 1978, not yet published) antiserum was prepared to VP 123 and to VP 119, the major glycoprotein region of HSV-2 infected cells, which were found to react type specifically with HSV-1 and HSV-2 infected and transformed cells in a membrane fluorescence test performed on live cells.

The other antigen of relevance to this study is the NVA-TAA (non-virion antigen - tumor-associated antigen) which was first described by Hollinshead et al (1972). This is a soluble membrane antigen which was first isolated from the supernate of sonicated vaginal, vulval, cervical and lip cancer cells in one of the fractions separated by sephadex gel filtration (Hollinshead et al, 1972; and Hollinshead and Tarro, 1973). It cross reacted in a complement fixation test with antiserum prepared in guinea pigs against semi-purified

HSV-2. The antigen was later prepared, by polyacrylamide gel separation, of the supernate obtained by stepwise disruption and sonication of HSV-2 infected cells (Hollinshead et al, 1973). Table 7.3 shows the proportion of sera from groups of patients with various malignancies that reacted with NVA-TAA. These results were repeated by Notter and Docherty (1976) and Hollinshead et al (1976).

Most sera reacted with antigen prepared from HSV-1 as well as from HSV-2 infected cells. Eighteen per cent reacted only with HSV-1 antigen and 5% only with HSV-2 antigen.

The presence of antibody to the NVA-TAA does not simply represent past infection with the herpes virus. Four sera from cervical cancer patients with no detectable antibody to herpes virus reacted with NVA-TAA (Hollinshead and Tarro, 1973). Similar proportions of the two groups of patients marked with an asterisk in Table 7.3 possessed HSV neutralising antibody and complement fixing antibody although they differed in the proportions possessing antibody to NVA-TAA.

Thus in summary, NVA-TAA is an antigen on HSV-1 and HSV-2 infected cells which cross reacts with an antigen on many squamous cell carcinomas.

The possible antigens that IgA anti-MA is directed against could thus be one or more of the membrane associated glycoproteins described by Vestergaard or VP 119 described by Courtney, or an antigen similar to NVA-TAA, i.e.

1. To an antigen common to HSV-1 infected cells and HSV-2 infected cells denoted CM 1 and 2 (common 1 and 2).

2. To antigens specific for HSV-2 infected cells, denoted TS-2 (type specific-2).
3. To a cross reacting antigen similar to NVA-TAA denoted Cr (cross reacting antigen) which cross reacts with a similar antigen on squamous carcinoma cells.

Evidence for detection of a cross reacting antigen between squamous cell carcinomas and HSV-2 infected cells by the IgA membrane fluorescence test on HSV-2 infected cells.

In addition to CM 1 and 2, TS-2 and Cr above, let TS-1 denote type specific antigen on HSV-1 infected cells. Patients who have had previous HSV-1 infection would possess anti CM 1 and 2 and anti TS-1. Patients who have had previous HSV-2 infection would possess anti CM 1 and 2 and anti TS-2, and patients who have had previous HSV-1 and HSV-2 infection would possess anti CM 1 and 2 and anti TS-1 and anti TS-2.

In Table 7.4 each group of patients in the study have been divided into two classes:

a) Those possessing type specific antibody to HSV-1 as determined by ELISA, and

b) Those possessing type specific antibody to HSV-1 and HSV-2.

Two groups of antigen on RK 13 cells infected with HSV-2 which were employed in the membrane fluorescence assay would be CM 1 and 2 and TS-2. Therefore among the Group 1 (British controls) class (a) patients only anti CM 1 and 2 antibodies would attach onto the test cells, whereas both anti CM 1 and 2 antibodies and anti TS-2 antibodies in Group 1 class (b) patients would attach onto the test cells. This explains the higher IgA anti-MA GMT of Group 1 class (b) patients in contrast to that observed among the Group 1 class (a) patients.

When one examines the IgA anti-MA GMT's of class (a) patients in Group 1, Group 2 (dysplasias), Group 3 (carcinomas in situ) and Group 4 (British untreated invasive carcinomas),

the class (a) carcinoma in situ patients and the class (a) untreated invasive carcinoma patients have a higher IgA anti-MA GMT than the class (a) controls or class (a) patients with dysplasia. Similarly, class (b) untreated invasive carcinoma patients have a higher GMT of IgA anti-MA than class (b) controls, class (b) dysplasia patients, or class (b) carcinoma in situ patients. Both class (a) and class (b) patients with non-genital squamous carcinoma (Group 5) had higher titres of IgA anti-MA than the class (a) and (b) controls respectively.

Suppose patients with squamous carcinomas possessed a Cr antigen, the groups of patients in Table 7.4 who would possess this antigen would be the patients with carcinoma in situ, invasive carcinoma (untreated) and non-genital squamous carcinoma, i.e. these patients would possess Cr antibodies which would attach to the test cells. This provides an explanation for the observation made before that class (a) and class (b) carcinoma in situ, untreated invasive carcinoma and non-genital squamous carcinoma patients had higher IgA anti-MA compared to class (a) and (b) dysplasias and controls. The class (b) carcinomas in situ patients do not strictly follow this pattern.

If the higher titres of IgA anti-MA in these patients was caused by the presence of anti Cr antibodies, one could predict that these levels would fall following treatment of the carcinoma and the destruction of Cr antigen by radiotherapy. This is seen among the class (b) treated invasive carcinoma patients (Group 6) who have a lower GMT of IgA anti-MA than the class (b) untreated invasive carcinoma patients. Once again similar to the class (b) carcinomas in situ patients

GROUP OF PATIENTS	CLASS OF PATIENTS	ANTIGENS IN VIVO WHEN PATIENT EXPERIENCED HSV INFECTION	ANTIBODIES	ANTIBODIES REACTING WITH HSV-2 INFECED CELLS	GMT OF IgA ANTI-MA
1. Controls Britain	a) Previous HSV-1 infection	CM 1 & 2	Anti CM 1&2	+	3.47
		TS 1	Anti TS 1	-	
	b) Previous HSV-1 & HSV-2 infection	CM 1 & 2	Anti CM 1&2	+	
		TS 1	Anti TS 1	-	8
		TS 2	Anti TS 2	+	
2. Dysplasia Britain	a) Previous HSV-1 infection	CM 1 & 2	Anti CM 1&2	+	3.48
		TS 1	Anti TS 1	-	
	b) Previous HSV-1 & HSV-2 infection	CM 1 & 2	Anti CM 1&2	+	
		TS 1	Anti TS 1	-	9.75
		TS 2	Anti TS 2	+	
3. Carcinoma in situ Britain	a) Previous HSV-1 infection	Cr	Anti Cr	+	
		CM 1 & 2	Anti CM 1&2	+	8
		TS 1	Anti TS 1	-	
	b) Previous HSV-1 & HSV-2 infection	Cr	Anti Cr	+	
		CM 1 & 2	Anti CM 1&2	+	8.61
		TS 2	Anti TS 2	+	
		TS 1	Anti TS 1	-	
4. Untreated invasive carcinoma Britain	a) Previous HSV-1 infection	Cr	Anti Cr	++	
		CM 1 & 2	Anti CM 1&2	+	5.48
		TS 1	Anti TS 1	-	
	b) Previous HAV-1 & HSV-2 infection	Cr	Anti Cr	++	
		CM 1 & 2	Anti CM 1&2	+	16.88
		TS 2	Anti TS 2	+	
		TS 1	Anti TS 1	-	

Table 7.4 Analysis of IgA anti-MA results according to the HSV type specific antibody distribution in British patients.

GROUP OF PATIENTS	CLASS OF PATIENTS	ANTIGENS IN VIVO WHEN PATIENTS EXPERIENCED HSV INFECTION	ANTIBODIES	ANTIBODIES REACTING WITH HSV-2 INFECTED CELLS	GMT OF IgA ANTI-MA
5. Non-genital squamous carcinoma. Britain	a) Previous HSV-1 infection	CM 1 & 2	Anti CM 1&2	+	7.51
		TS 1	Anti TS 1	-	
		Cr	Anti Cr	++	
	b) Previous HSV-1 & HSV-2 infection	CM 1 & 2	Anti CM 1&2	+	10.77
		TS 2	Anti TS 2	+	
		Cr	Anti Cr	++	
		TS 1	Anti TS 1	-	
6. Treated invasive carcinoma Britain	a) Previous HSV-1 infection (Previous Cr)	CM 1 & 2	Anti CM 1&2	+	6.96
		TS 1	Anti TS 1	-	
	b) Previous HSV-1 & HSV-2 infection (Previous Cr)	CM 1 & 2	Anti CM 1&2	+	8.00
		TS 2	Anti TS 2	+	
		TS 1	Anti TS 1	-	
7. Other genital malignancies (non-squamous) Britain	a) Previous HSV-1 infection	CM 1 & 2	Anti CM 1&2	+	3.03
		TS 1	Anti TS 1	-	
	b) Previous HSV-1 & HSV-2 infection	CM 1 & 2	Anti CM 1&2	+	8.00
		TS 2	Anti TS 2	+	
		TS 1	Anti TS 1	-	
8. Non-squamous extra-genital malignancies Britain	a) Previous HSV-1 infection	CM 1 & 2	Anti CM 1&2	+	3.62
		TS 1	Anti TS 1	-	
	b) Previous HSV-1 & HSV-2 infection	CM 1 & 2	Anti CM 1&2	+	4.67
		TS 1	Anti TS 1	-	
		TS 2	Anti TS 2	+	

Table 7.4 (cont'd) Analysis of IgA anti-MA antibody results according to the HSV type specific antibody distribution in British patients.

the class (a) treated invasive carcinomas do not have a GMT which strictly falls into the pattern. This is to be expected in any biological system, and does not necessarily detract from the argument. However, in support of the above hypothesis is the fact that among the Group 4 patients, none of the seven patients who later developed recurrence of their malignancy showed a decline in titre of IgA anti-MA within ten weeks from the onset of treatment whereas nine of twenty (45%) patients who progressed without recurrence did (Table 6.45,)

Class (a) and (b) patients with non-squamous genital malignancies (Group 7) and non-genital non-squamous malignancies (Group 8) had IgA anti-MA GMT's similar to class (a) and (b) controls (Group 1). This would have been expected on the hypothesis that Cr antigen is present only on squamous carcinoma cells.

A similar picture is seen among class (a) and (b) patients among Sri Lankan and Malawian controls, and invasive carcinoma patients (Table 7.5). The group (b) Sri Lankan and Malawian invasive carcinoma patients possess much higher titres of IgA anti-MA than their respective class (b) controls.

If one postulates a cross reacting antigen between HSV infected cells and squamous carcinoma cells, one must also consider that during an episode of herpetic infection the infected cells <u>in vivo</u> may express such an antigen. It is possible even so that the amount of cross reacting antigen is extremely small and not sufficient an antigenic stimulus compared to the quantity expressed on a malignant cell.

GROUP OF PATIENTS	CLASS OF PATIENTS	ANTIGENS IN VIVO WHEN PATIENTS EXPERIENCED HSV INFECTION	ANTIBODIES	ANTIBODIES REACTING WITH HSV-2 INFECTED CELLS	GMT OF IgA ANTI-MA
1. Controls Sri Lanka	a) Previous HSV-1 infection	CM 1 & 2	Anti CM 1&2	+	4.00
		TS 1	Anti TS 1	-	
	b) Previous HSV-1 & HSV-2 infection	CM 1 & 2	Anti CM 1&2	+	5.66
		TS 1	Anti TS 1	-	
		TS 2	Anti TS 2	+	
2. Untreated invasive carcinomas Sri Lanka	a) Previous HSV-1 infection	CM 1 & 2	Anti CM 1&2	+	3.48
		TS 1	Anti TS 1	-	
		CY	Anti CY	+	
	b) Previous HSV-1 & HSV-2 infection	CM 1 & 2	Anti CM 1&2	+	29.49
		TS 1	Anti TS 1	-	
		TS 2	Anti TS 2	+	
		CY	Anti CY	+	
	c) Previous HSV-2 infection	CM 1 & 2	Anti CM 1&2	+	20.16
		TS 2	Anti TS 2	+	
		CY	Anti CY	+	
3. Controls Malawi	a) Previous HSV-1 infection	CM 1 & 2	Anti CM 1&2	+	6.86
		TS 1	Anti TS 1	-	
	b) Previous HSV-1 & HSV-2 infection	CM 1 & 2	Anti CM 1&2	+	13.45
		TS 1	Anti TS 1	-	
		TS 2	Anti TS 2	+	
4. Untreated Invasive carcinomas Malawi	a) Previous HSV-1 infection	CM 1 & 2	Anti CM 1&2	+	5.94
		TS 1	Anti TS 1	-	
		CY	Anti CY	+	
	b) Previous HSV-1 & HSV-2 infection	CM 1 & 2	Anti CM 1&2	+	37.03
		TS 1	Anti TS 1	-	
		TS 2	Anti TS 2	+	
		CY	Anti CY	+	

Table 7.5 Analysis of IgA anti-MA results of Sri Lankan and Malawian patients and controls according to the distribution of HSV-type specific antibody.

This would also explain the reason why the difference in antibody titre between squamous cancer bearing patients and non-tumor bearing patients is present, but not remarkably large. Nevertheless, this preliminary evidence for a cross reacting antigen carries an important implication. In patients with invasive cervical carcinoma, antibody to cross reacting antigen may contribute towards a false result of elevated antibody titres to HSV antigens. It may therefore be of value to identify which of the antibody assays employing herpes simplex antigens are able to detect such anti-Cr antibodies. This may in particular be relevant to assays which test for antibody to surface antigens of HSV infected cells. Thus patients with non-genital squamous carcinomas should form an important control group in such studies.

Persistence of IgA anti-MA in patients after treatment for carcinoma of the cervix.

The key to the reason for this persistence lies in the prevalence of these antibodies observed among patients with genital herpes. In contrast with IgA anti-VCA antibodies which were present for two to three months after clinical infection, IgA anti-MA persisted in every serial sample of serum from these sixteen patients with genital herpes. This period of follow up extended to one year and three months in three patients who during that period had no apparent recurrences. Furthermore, attempts to isolate virus from these patients during the period of follow up were unsuccessful. The current concepts and unknown factors concerning herpes simplex virus latency were described in Chapter 2, page 66 , and the serial, sequential formation of α, β and γ polypeptides during viral infection were also explained.

Herpes simplex virus has been isolated from the trigeminal ganglia and sacral ganglia of human cadavers (Lonsdale et al, 1979). HSV-2 was isolated from the sacrosciatic ganglia of mice infected with HSV-2 via their foot pads (Stevens and Cooke, 1973). Possibly the neural ganglion has evolved as the most advantageous site for viral survival as a means of escape from the immune system. The interesting question then which arises is which antigenic stimulus is responsible for maintaining levels of IgA anti-MA over extended periods at levels similar to those observed during convalescence. It is known that IgA is catabolised at a higher rate than IgG (Tomasi, 1972) and that the half-life of IgA is six days (Fenner and Blanden, 1975). Thus it would

be expected that continuous stimulation of the immune system is required for the maintenance of IgA levels over long periods. This antigenic stimulus could come from one of two sources:

1. From low grade viral production, or
2. From viral expression which falls short of production of viral progeny, at a site which is available to the immune system.

If the first possibility had been correct, it could be expected that IgA anti-VCA antibodies, i.e. antibodies directed against the late viral proteins (γ polypeptides) too would persist - which was not the finding in this study. It therefore appears that the second possibility is the more likely one. By viral expression which falls short of production of viral progeny is meant a state in which:

a) There is no DNA replication
b) There is no inhibition of host cells' macromolecular synthesis, i.e. translation of viral DNA is arrested before its β polypeptides are formed. This is necessary for the long-term survival of the virus in this state.

Therefore, by a process of elimination, viral DNA could be translated up to the stage of forming α polypeptides or early polypeptides. From the experiments summarised in Table 4.8 of the Introduction (page 90) it was seen that at least some of the membrane antigens on HSV infected cells were not dependent on DNA replication for their formation. Furthermore, the experiments of Courtney (page 94) demonstrated membrane antigens on the surface of transformed cells in which it is known that viral expression is arrested before DNA

replication occurs. Thus it seems plausible to suggest that the virus lies expressing its α polypeptides in an apparently healthy host cell which unlike the cells within the nerve ganglion are freely available to the immune system. In this manner the early viral proteins which are expressed on the cell surface would be able to stimulate the immune system. This leads to the next important question which is the site at which these cells carrying "latent" virus lie. It is possible that they lie at the site of original infection, which is the external genitalia, vagina and the cervical epithelium. The evidence from this study on the effects of radiotherapy on antibody levels supports the possibility of the virus lying in the cervical epithelium. A significantly higher proportion of the patients with cervical carcinoma showed a rise in IgG anti-MA to HSV-2 infected cells following radiotherapy. This contrasted with the proportion who demonstrated rise in haemagglutination-inhibition antibody to two other common viruses - rubella and measles, i.e. this comparison suggested that the rise in titre of IgG anti-MA was caused by release of viral antigen following radiotherapy and was not due to a non-specific general stimulus to antibody levels. Thus the herpes specific IgA antibody results on patients with genital herpes may be interpreted as follows: That whereas IgA anti-VCA antibodies are markers of recent productive infection, IgA anti-MA antibody is a marker of latent viral expression in cells available to the immune system. It is therfore suggested that the virus lies latent at two sites: the nerve ganglion and a local site possibly the cervical epithelium. Sri Lankan and British patients with cervical carcinoma were

treated by radiotherapy. Even if radiotherapy is responsible for the destruction of virus and viral antigens at the local sites, some patients may harbour a reservoir of virus in the sacral ganglia which could re-infect local sites. The initial destruction of virus and viral antigen at local sites could explain the initial fall in level of IgA anti-MA observed directly following radiotherapy in patients who did not develop recurrences. From Table 6.45, although nine of twenty (45%) showed a fall in level of IgA anti-MA up to ten weeks after the onset of treatment, only four of fifteen (25%) demonstrated a fall in level at the end of the period of follow up. It may therefore be deduced that in some patients local sites were re-infected with virus and IgA anti-MA levels returned to previous levels.

The high prevalence of IgA anti-MA in patients with cervical anaplasia

The high prevalence of IgA anti-MA antibody in patients with cervical anaplasia must be interpreted in the face of evidence that:

a) the IgA anti-MA response is more type specific than the IgG anti-MA response

b) IgA anti-MA may be a marker of the presence of latent virus expressing part of its genome

c) IgA anti-MA may be in part directed against an antigen(s) common to HSV-2 infected cells and squamous carcinoma cells.

Thus, the reason for the high prevalence of IgA anti-MA in patients with cervical anaplasia may be in part an indication of a higher incidence of HSV-2 infection in patients with cervical anaplasia. This is supported by the prevalence of HSV-2 specific antibody detected by ELISA in a significantly higher proportion of patients than in controls. Furthermore, it is tempting to speculate that patients in whom an IgA anti-MA response was detected not only were previously infected with HSV-2 but concurrently harboured latent virus which was expressing some of its genome. The increased IgA anti-MA titres with disease progress may either be a reflection of increased expression of HSV-2 related antigens or a reflection of IgA anti-MA directed against the antigen common to HSV-2 infected cells and squamous carcinoma cells. Such a tumor specific cross reacting antigen would be expressed in larger quantity as the tumor increased in mass.

IgA anti-MA in British patients with malignancies other than squamous carcinoma of the cervix.

Few studies have examined the prevalence of HSV-2 antibodies in patients with malignancies other than cervical carcinoma. In those which have, either the numbers have been small (Rawls et al, 1968 and 1969; Aurelian et al, 1973) or the type of neoplasm has been unspecified by the authors (Freymouth et al, 1975; Sprecher-Goldberger et al, 1970; Aurelian et al, 1973). In this study, patients with malignant disease other than squamous carcinoma of the cervix were classified into three separate groups, these being a) patients with other genital malignant disease, b) patients with extra-genital non-squamous malignant disease, and c) patients with non-genital squamous malignancies.

On analysing the IgA anti-MA results of forty-three British patients whose HSV-1 and HSV-2 type specific antibody had been tested by ELISA it was found that a titre of IgA anti-MA greater than or equal to 1:8 provided evidence of previous HSV-2 infection (page 191). It was also suggested above that the presence of IgA anti-MA may signify the presence of latent HSV-2 virus. Table 7.6 contrasts the prevalence of HSV-2 specific antibody (ELISA) and IgA anti-MA at a titre of greater than or equal to 1:8 in British patients with cervical anaplasia and British patients with malignancies other than cervical anaplasia.

Although similar proportions of patients with other genital malignancies possessed HSV-2 specific antibody by ELISA, a significantly higher proportion of patients with

cervical anaplasia possessed IgA anti-MA at tires of greater than or equal to 1:8. This finding implies that whereas evidence for previous HSV-2 infection does not distinguish these two groups of patients, evidence for current expression of latent HSV-2 does. It must however be born in mind that if a cross reacting antigen exists between squamous carcinoma cells and HSV-2 infected cells, antibody to such an antigen may confuse the results of IgA anti-MA in patients with carcinoma in situ and invasive carcinoma, but not in patients with dysplasia.

Compared with patients with cervical anaplasia, a significantly lower proportion of patients with extra-genital non-squamous malignancies had been previously infected with HSV-2. An even lower proportion of these patients showed evidence of expression of latent HSV-2 virus.

Although a smaller proportion of patients with non-genital squamous carcinoma had been previously infected with HSV-2 in comparison with patients with cervical anaplasia, this difference was not significant. However a higher proportion of patients with non-genital squamous carcinoma had IgA anti-MA greater than or equal to 1:8 when compared with patients with cervical anaplasia. This discrepancy may be due to IgA anti-MA being in part formed against an antigen(s) common to HSV-2 infected cells and squamous carcinoma cells.

Thus the subdivision of patients with malignancies other than cervical carcinoma into three broad groups provided revealing results. If a causal relationship between the virus and cervical anaplasia is to be upheld, it is necessary to

Table 7.6 Previous infection by HSV-2 (ELISA), and concurrent infection by latent HSV-2 (IgA anti-MA ⩾1/8), in British patients with malignancies other than squamous cervical carcinoma.

PATIENT GROUP	CONCURRENT EXPRESSION OF LATENT HSV-2 (IgA ANTI-MA ⩾1/8)		PREVIOUS INFECTION BY HSV-2 (ELISA)	
Other genital malignancies	$\frac{9}{33}$ (27%)		$\frac{11}{21}$ (52%)	
		P < .025		P > .05
Cervical anaplasia	$\frac{47}{88}$ (53%)		$\frac{65}{104}$ (63%)	
Extra-genital non-squamous malignancies	$\frac{8}{37}$ (21%)		$\frac{9}{29}$ (31%)	
		P < .005		P < .005
Cervical anaplasia	$\frac{47}{88}$ (53%)		$\frac{65}{104}$ (63%)	
Non-genital squamous carcinoma	$\frac{16}{24}$ (66%)		$\frac{9}{21}$ (43%)	
		P > .05		P > .05
Cervical anaplasia	$\frac{47}{88}$ (53%)		$\frac{65}{104}$ (63%)	

identify an HSV-2 related variable which distinguishes patients with cervical anaplasia not only from matched controls but from patients with other malignancies and from patients who have previously had genital herpes but who continue to possess healthy cervices. It is therefore significant that evidence for the presence of concurrent infection by latent HSV-2 distinguishes patients with cervical anaplasia from patients with other genital malignancies. It is also significant and worthy of further investigation that patients with other squamous carcinoma are not distinguished from patients with cervical anaplasia by either evidence for previous or concurrent infection by HSV-2.

It is of interest that in contrast to Aurelian et al (1973) who did not detect AG-4 antibodies in a small number of patients with other malignancies (mostly unspecified), Notter and Docherty (1976) detected AG-4 antibodies in five of eight (63%) patients with non-genital squamous malignancies. In the context of results in the study reported in this thesis, this finding assumes greater significance, for according to Aurelian et al (1977), AG-4 antigen is detected on the surface as well as cytoplasm of HSV-2 infected cells. Could the AG-4 assay also be detecting antibody to cross reacting antigen? In contrast in the study by Notter and Docherty only four of twelve (33%) patients with extra-genital non-squamous malignancies possessed AG-4 antibodies. These results are very similar to those in the present study.

Both AG-4 and VP 134 (Anzai et al, 1975) are antigens produced early in the infectious cycle and may therefore belong to the group of α polypeptides. Antibodies to both these anti-

gens distinguish patients with cervical anaplasia from controls. However it is unfortunate that the prevalence of antibody to AG-4 and VP 134 have not been studied in patients with genital herpes.

In the studies reported in this thesis, the prevalence of IgA anti-MA in patients with cervical anaplasia and patients with genital herpes was similar up to the period that these patients were followed up. However if a longer prospective study of patients with genital herpes shows that IgA anti-MA persists only in women who later develop cervical anaplasia, this finding would strongly support the hypothesis that HSV-2 is causally related to cervical anaplasia.

C. ANTIBODIES TO HSV-2 TYPE SPECIFIC ANTIGEN BY ELISA

Of all the antibody assays that were employed in this study, the most sensitive for assaying previous infection by HSV-2 was ELISA. Although the IgA anti-MA assay was more type specific than the IgG anti-MA assay, the interpretation of antibody titres in patients with cervical anaplasia is confused by the finding that the assay may be recognising antibodies directed against an antigen which is common to HSV-2 infected cells and squamous carcinoma cells. Compared with the ELISA technique, the CF test for HSV-1 and HSV-2 specific antibody gave more false negative readings.

By ELISA, a higher proportion of patients with cervical anaplasia from Britain, Sri Lanka and Malawi had HSV-2 specific antibodies than matched controls. This difference was statistically significant except among British patients with dysplasia. These results are similar to those obtained in the retrospective studies on patients with cervical anaplasia in the USA, West Indies, Europe, India and South Africa (Table 1.4). The studies reported in this thesis confirm the results of two previous studies conducted in Britain by Skinner et al showing an association between HSV-2 and cervical carcinoma. Furthermore, the results show the virus is associated with cervical carcinoma in Sri Lanka and Malawi where no previous studies had been conducted. Patients with cervical anaplasia who had no detectable HSV-2 specific antibodies.

Approximately 27-48% of all patients with cervical anaplasia had no detectable HSV-2 specific antibodies by ELISA, the proportion being highest among patients from Sudan

(Table 6.15). The view was expressed by Rawls et al in 1977 that HSV-2 specific antibody had been underestimated among women with cervical anaplasia. This was believed in part to be due to techniques employed at the time not being sufficiently sensitive. Since then ELISA for HSV-2 specific antibody has been developed which in addition employs type specific antigen. From a comparison of fifty-eight sera in this study which were tested by both CF and ELISA, it was estimated that ELISA gave a false negative rate for HSV-2 specific antibody of 17%. Thus, even if for the sake of argument the benefit of this 17% error is given to the proportion of patients with no detectable HSV-2 specific antibodies, there are still 10-31% of patients with cervical anaplasia with no HSV-2 specific antibodies. It is likely that this is a real lack of HSV-2 antibodies and not attributable to the insenstivity of laboratory techniques. Of the studies cited in Table 1.4 those conducted in Taiwan, Japan, New Zealand, Colombia, and Israel did not show an association between HSV-2 and carcinoma of the cervix. However even in those which did, most showed as in this study, a proportion in which HSV-2 antibodies were not detectable. The proportion of British, Sri Lankan and Malawian patients who lacked both HSV-1 and HSV-2 specific antibody was only 1.7%, i.e. a high proportion of patients with cervical anaplasia in this series, possessed HSV-1 specific antibody. This presents the possibility that in the aetiology of cervical carcinoma:

1. HSV-1 too is causally related,
2. Agents other than HSV-1 and HSV-2 are causally related, or
3. That aetiology could vary with age or geographic distribution.

1. HSV-1 as an aetiologic agent of cervical carcinoma.

HSV-1 has been shown to possess as much oncogenic potential as HSV-2 as demonstrated by transformation experiments and the oncogenicity of HSV-1 transformed cells in animals (Table 1.5). Wyburn-Mason (1957) reported six patients between the ages of sixty and seventy in whom oral carcinoma developed at sites at which recurrent herpetic lesions occurred. It was proposed as a possible causative agent of cervical carcinoma in Japanese by Kawana et al (1976), when a much lower prevalence of AG-4 antibodies was detected in Japanese sera than in American sera. Fifty per cent of genital herpes simplex isolates made in Japan were HSV-1. The predominant virus of genital infections in Sri Lanka and Malawi is not known. However in the Department of Virology at St. Thomas', of nine hundred forty genital isolates made over a two year period, only six (0.6%) were HSV-1. This provides clear evidence that HSV-2 is by far the commonest virus of genital infection in the population from which the British patients with cervical anaplasia in this study were drawn. It therefore does not appear likely that HSV-1 could be causally related to the tumor in the British patients in this study.

Agents other than HSV-1 or HSV-2 which could be aetiologically related to cervical carcinoma.

Countries in which no association between HSV-2 and cervical carcinoma was demonstrated were cited in Table 1.4. Yet the epidemiological features of the malignancy strongly suggest that it is related to a sexually transmitted agent.

It is possible therefore that there are mulitple aetiologic agents and that they play roles of varying importance in different geographic areas. Thus while in a single geographic area, one agent may be responsible for the majority of cases of cervical carcinoma, other agents may be responsible for a small proportion of tumors in that area. An analogous situation is seen in the case of Burkitt's lymphoma (BL). BL occurs sporadically but rarely in areas outside the endemic areas of Africa and New Guinea. These non-endemic cases differ slightly in age distribution and tumor sites, but are indistinguishable in histology, cytology and their clinical response to chemotherapy, from endemic BL seen in Africa and New Guinea. The majority of these non-endemic BL do not show an association with the Epstein-Barr virus (EBV), but a small proportion (8-17%) do (Epstein and Achong, 1977). No agent has been found up to date which is aetiologically related to these non-EBV related BLs

The possibility also exists that another agent is the real aetiologic agent of cervical carcinoma and that HSV-2 is one of its co-variables. There is some evidence from the studies reported in this thesis to support this possibility. In the cohort analysis on British patients and controls which examined the prevalence of HSV-2 specific antibody in cohorts born during ten year periods from 1900 to 1960, a higher prevalence of HSV-2 specific antibody was not observed in all cohorts of patients in comparison with controls. If the virus was causally related, such a constant higher prevalence would be expected regardless of the year of birth. A steep rise in the prevalence of HSV-2 specific antibody was observed among cohorts of patients born between 1921 and 1930, thirty

years before a similar rise in prevalence was observed in the cohort of controls born between 1951 and 1960. This could well be interpreted as being a marker of greater promiscuity among patients with cervical anaplasia compared with controls, and that HSV-2 was a co-variable of another sexually transmitted agent. Were it possible to determine antibody to this agent, there may have been a higher prevalence of such antibody in all cohorts of patients in the study compared with controls. On the other hand, a high prevalence of HSV-2 specific antibodies was detected in both patients and controls born between 1951 and 1960. This coincides with the higher incidence of genital herpes observed recently in venereal diseases clinics in England (Skinner et al, 1978). If HSV-2 is causally related to cervical carcinoma, it may then be predicted that this would result in a similar rise in the incidence of cervical anaplasia.

The cross sectional studies cited in the Historical Survey of this thesis indicate that chlamydiae, mycoplasma, gonococci, cytomegalovirus and human papilloma virus may be associated with cervical anaplasia (pages 49-59). The association may be of a causal, co-factor or a synergistic nature. A noticeable deficiency in cervical carcinoma studies is that researchers have not taken adequate notice of this possibility. It is therefore suggested in this thesis that future studies should examine evidence for all such aetiologic agents in a single groups of patients and controls, preferably drawn from different geographic areas. Such longitudinal studies would provide more information than isolated cross sectional studies.

3. The possibility that the aetiology of cervical carcinoma could vary according to geographic area or age.

British patients with cervical anaplasia who were above sixty years when the lesion was detected had a noticeably lower prevalence of HSV-2 specific antibody (37%) than those who were younger (66-80%). This difference is unlikely to be due to a loss of antibody with age, since in the above cohort analysis, cohorts of controls born between the years 1900-1910 and 1941-1950 had a similar prevalence of HSV-2 specific antibody. This suggested that controls in this study who were over the age of sixty years had not suffered loss of antibody as detected by ELISA. Malawian patients too, who were over sixty years at the time of tumor detection had a lower prevalence of HSV-2 specific antibodies (60%), in contrast with those in whom the tumor was detected at an earlier age (78%). However this difference was not observed among Sri Lankan patients (83% and 65% respectively).

The pathological changes supposedly induced by EBV are among other factors age dependent. BL develops usually following childhood infection by EBV, and infection mononucleosis from primary EBV infection in young adults (Epstein and Achong, 1977). In addition there is a difference in the age distribution of pateints with EBV related and EBV unrelated BL. In New Zealand where no association was observed between HSV-2 and cervical carcinoma, only 13% of patients were aged forty years or younger. In contrast in Houston, where an association was detected between the virus and the malignancy, 22% of patients were forty years or younger (Rawls et al, 1970).

It is possible that at the time in New Zealand the majority of cases of cervical carcinoma occurred in older women and were aetiologically related to an agent other than HSV-2. This may also be true of populations in other countries. Unfortunately few studies on HSV-2 antibodies in patients with cervical anaplasia state the age distribution of patients in the study. Thus, this information could not be derived from studies conducted on populations in Formosa, Colombia, Taiwan, Japan and Israel. In all these countries no association was detected between the virus and the tumor. For this reason, these populations would be ideal for the study of other possible aetiologic agents in cervical carcinoma, as well as a study of variation in aetiology according to age.

CRITERION NUMBER 3

THE VIRUS SHOULD BE SHOWN TO CAUSE CERVICAL LESIONS WHICH SHOULD OCCUR WELL BEFORE THE ONSET OF NEOPLASTIC CHANGE. AND A HIGHER INCIDENCE OF THE TUMOR SHOULD BE OBSERVED IN THESE WOMEN.

Indirect evidence for this criterion is provided in the studies reported in this thesis as it confirmed previous findings that the average age of patients presenting with genital herpes (23)was younger than that of patients presenting with dysplasia (35), carcinoma in situ (37) and invasive carcinoma (52).

Catalano and Johnson (1971) demonstrated by kinetic neutralisation, HSV-2 specific antibody in the sera of five of fourteen women who developed carcinoma in situ one to eight years after that particular sample of serum had been collected. Although such retrospective studies are possible in instances where banks of serum are maintained, the studies of Sogbetun et al (1979) question the validity of concluding the HSV-2 specific antibody always results from a previous cervical infection. These workers examined four hundred twenty-two sera from children and young adults living in Ibaden, Nigeria. Eleven per cent of children in the three to five year age group and 14% of children in the six to ten year age group possessed HSV-2 specific antibody. A microneutralisation test was employed. The authors were of the opinion that those HSV-2 antibodies were acquired by non-venereally transmitted HSV-2 infection. The mechanism of such transmission they suspected was by means of shared towels. bed clothes and beds, among people living in poor socio-economic conditions. Similar

studies had been previously reported by Osoba and Alausa (1974) who found that gonococcal vulvo-vaginitis in young girls in Ibaden was relatively frequent, and not caused by sexual contact. Gonococci like HSV are extremely labile organisms. They demonstrated that they survived long enough for such transmission to be possible when deposited on cloth exposed to humid air during the rainy season in Nigeria. Since sera for studies reported in this thesis were drawn from low socio-economic groups in three third world countries, it is recognised that in a small proportion of patients the presence of HSV-2 specific antibody may not have been an accurate index of previous cervical infection by HSV-2. In contrast to the Ibaden study, Nahmias et al (1970c) found in an American population that HSV-2 specific antibodies were acquired after the age of fourteen years, and coincided with the onset of sexual activity. It is likely that in Britain HSV-2 antibodies are acquired similarly and therefore yield more accurate information as to previous cervical infection by HSV-2. Nevertheless, the most concrete proof of cervical HSV-2 infection is from the isolation of virus from clinical lesions or from apparently healthy cervices. Hence the most reliable evidence for this criterion must be sought in prospective rather than retrospective studies. Had more adequately controlled and prolonged prospective studies been conducted over the last thirteen years following establishment of the relationship between the virus and the tumor, more progress may have been made as to the aetiological significance of HSV-2 in cervical carcinoma.

CRITERION NUMBER 4

SIMILAR VIRUSES OF THE HERPES GROUP SHOULD CAUSE NATURALLY OCCURRING MALIGNANCIES IN OTHER ANIMALS.

There is no malignancy in animals of viral or non-viral aetiology which is analogous to squamous carcinoma of the cervix. However, other members of the herpes group of viruses induce malignancies in animals, and the Epstein-Barr virus (EBV) has been strongly linked to two human malignancies - Burkitt's lymphoma (BL) and nasopharyngeal carcinoma (NPC). These herpes induced malignancies have been described in the Historical Survey (page 32-35). It is appropriate that cervical carcinoma should be compared and contrasted with them.

Among the known herpes virus induced malignancies in animals, none occur in epithelial cells and in this respect nasopahryngeal carcinoma is the closest analoge to squamous carcinoma of the cervix. Studies in patients with NPC show that approximately 90% possess IgA antibody to VCA of EBV and 73% possess IgA to the diffuse component of early antigen (Henle and Henle, 1976; Ho et al, 1976). IgA anti-VCA titres in both studies ranged from 1:10 to 1:640. These titres increased with progress of the tumor and declined after treatment. The only EBV related antibody which did not show decline following treatment was antibody to Epstein-Barr virus nuclear antigen (EBNA). In contrast to patients with NPC, only 28% of patients with BL possessed IgA anti-VCA to EBV.

Thus, of the two human malignancies associated with EBV, NPC a tumor of epithelial cells rather than BL a tumor of the

lymphoproliferative system, elicits a marked IgA host response. However, a parallel IgA response to VCA of HSV-2 was not observed among patients with cervical anaplasia in either this study or that conducted by El Falaky and Vestergaard (1977). Nevertheless, a significantly higher proportion of patients with cervical anaplasia in this study had IgA antibodies to the membrane antigen (MA) of HSV-2 infected cells, in titres comparable with those observed in convalescent sera of patients infected with HSV-2. Thus these results are similar to the IgA results of patients with NPC, but not identical. The prevalence of IgA antibody to membrane antigens of EBV infected cells have not been studied in patients with NPC.

Desgranges et al (1977) detected IgA anti-VCA to EBV in the saliva of 50% of patients with NPC. They also detected IgA anti-VCA to EBV in the plasma cells surrounding the epithelial tumor cells. Similar studies on HSV-2 specific IgA anti-VCA or anti-MA in the cervical mucus of patients with cervical carcinoma have not been carried out. However, patients with cervical anaplasia possessed significantly higher levels of total IgA in their cervical mucus (Coughlan and Skinner, 1977). Thus both the humoral and local IgA mechanism are stimulated by both NPC and carcinoma of the cervix. The presence of IgA anti-MA to HSV-2 infected cells in the sera of patients with cervical carcinoma is indirect evidence that the antigenic stimulus originates from an epithelial surface, i.e. the cervical epithelium.

Although viral antigens have not been demonstrated in cervical carcinoma tissue, both Pacsa et al (1977) and Royston and Aurelian (1970b) claim to have demonstrated herpes antigens

in the exfoliated cervical cells of patients with cervical carcinoma (pages 42 and 43). In the study by Pacsa, these antigens were detected in the exfoliated cells of forty-five of forty-nine (92%) of women with cervical carcinoma. It is possible that some environmental factor within the milieu of the cervix stressed the virus in these cells to express more polypeptides. Malignant lymphoma in monkeys provides a similar example of a herpes virus induced tumor in which no viral antigens can be demonstrated in cells obtained directly from the tumor. But a small proportion of these cells express viral antigens when transplanted into tissue culture. Similarly, a small proportion of EBV transformed cells which usually express only nuclear neoantigen EBNA and the membrane antigen LYDMA, are stressed into producing membrane antigen, early antigen and VCA on being treated with halogenated pyrimidines (Hampar et al, 1972), EBV genome has been detected in all NPC and all endemic tumors. Similarly the genome of herpes virus siamiri, and herpes virus ateles have been detected in malignant lymphomas in monkeys. HSV-2 DNA has so far been demonstrated only in the tumor tissue from one cervical carcinoma (Frenkel et al, 1972). They used DNA-DNA hybridisation which measured the rate of reassociation between radioactively labelled HSV-2 DNA and a surgically removed cervical carcinoma cell DNA. The segment of DNA detected represented 39% of the HSV-2 genome. Attempts made by other workers to repeat these findings were unsuccessful. Thus zur Hausen et al (1974) used a technique of cRNA-DNA hybridisation and searched for HSV-2 DNA in ten human cervical carcinomas. This technique was estimated to be adequate to detect one genome equivalent per cell. Schulte-Holthausen (1975) used a similar technique which was sufficiently sensitive

to detect 20% of a genome equivalent per cell but failed to detect HSV-2 DNA in forty-six cervical carcinoma biopsies. Pagano (1975) used DNA-DNA renaturation kinetics which he estimated were sufficiently sensitive to demonstrate 25% of the HSV-2 genome. Considering that in SV-40 transformed cells as little as one-third of a genome (2.6×10^6) is sufficient to maintain the transformed state, if the portion of the HSV-2 genome in cervical carcinoma cells is as little as this, a technique ten times more sensitive than present techniques would be required to detect one-hundredth part of the HSV-2 genome. It is noteworthy that by the same techniques as used by zur Hausen et al (1974), four to one hundred thirteen genome equivalents of EBV have been demonstrated in Burkitt's lymphoma cells (Pagano, 1974). However, at the herpes virus workshop held at Cambridge, England in 1978, J.K. McDougal presented encouraging preliminary work, where by a technique of in situ hybridisation he was able to demonstrate HSV RNA in 60% of biopsies of cells undergoing pre-malignant change. This work has not yet been published.

The temperature dependence of the Lucké virus and the geographical prevalence of BL and NPC demonstrate the extent to which the expression of oncogenic herpes viruses are governed by host, environmental and other co-factors. Although cervical carcinoma is a widespread tumor, it is possible that such selective factors may operate within the milieu of the cervix.

Although in areas where BL is common, antibodies to EBV are acquired at a very young age and nearly 50% of individuals are doubly infected with both malaria and EBV, the number

developing tumors is small. Suggested reasons for this include genetic predisposition, very early infection or a particular sequence in the timing of infection in relation to acquisition of malaria (Epstein, 1978b). The recent Ugandan prospective study (de The et al, 1978) showed that children with anti-EBV-VCA antibodies of two doubling dilutions or higher than control levels carried a thirty times higher risk of developing the tumor, i.e. some host factor made these children respond to the virus in a different manner. Similarly host factors such as the state of the cervical epithelium (metaplastic or non-metaplastic), HLA type, concurrent infection with other organisms, and the immune status of the patient may be of importance in the development of cervical carcinoma.

The association of the guinea pig herpes virus with an RNA virus, and the activation of leukaemia virus by the Marek's disease virus is of interest as Duff and Rapp (1975) observed that C-type virus was released from HSV-2 transformed 3Y3 cells after serial passage in culture. Similarly, infection of Balb/C mouse cells with UV irradiated HSV-1 and HSV-2 resulted in activation of an endogenous C-type virus (Hampar et al, 1976). This presents the possibility that induction of C-type virus synthesis may be a step in the induction of oncogenic transformation (Huebner and Todaro, 1969).

CRITERION NUMBER 7

INDIVIDUALS PROTECTED FROM DEVELOPING GENITAL HERPES INFECTION SHOULD DEMONSTRATE A LOWER INCIDENCE OF CERVICAL ANAPLASIA IN LATER YEARS

One of the aims of this study was to determine whether previous infection with HSV-1 protected from cervical anaplasia. The answer to this question is important as such evidence may provide a basis for attempts at immunprophylaxis. The evidence was examined from two different angles:

A. By attempting to determine where there is an inverted case control prevalence of HSV-1 specific antibody, i.e. do more controls than patients with cervical anaplasia possess only HSV-1 specific antibody without HSV-2 specific antibody as a result of them being protected from subsequent HSV-2 infection?

B. If previous HSV-1 infection does not protect from subsequent HSV-2 infection, does it protect from malignant transformation by HSV-2. If this occurs then one would expect the proportion of patients with cervical anaplasia who had only HSV-2 specific antibody to be greater than the corresponding proportion in the general population.

A. The inverted case control prevalence of HSV-1 specific antibody

When compared with patients with cervical anaplasia from Britain, Sri Lanka and Malawi, a higher proportion of matched controls possessed only HSV-1 specific antibody without HSV-2 specific antibody. This difference was significant in the comparison between Malawian and British patients with invasive

carcinoma and British patients with carcinoma _in situ_ and their respective controls. These findings confirm those of Skinner _et al_ (1977). If this inverted case control prevalence of HSV-1 antibody alone is to be interpreted as being a significant one from the view-point of the protective effect of previous HSV-1 infection, certain assumptions need to be made. That patients and controls are equally exposed to HSV-2 infection and that more patients possess both HSV-1 and HSV-2 specific antibody because more of them succumbed to HSV-2 infection. The greater susceptibility to HSV-2 infection among patients would then presumably be due to:

a) being infected by HSV-1 after they were infected by HSV-2, which is possible but unlikely, and
b) the inability of the immune mechanism of the patients to mount a response during previous HSV-1 infection which was sufficient to protect from subsequent HSV-2 infection.

The alternative explanation for the inverted case control prevalence of only HSV-1 specific antibody is that the control population comprises one which is less exposed to HSV-2 than the patient population. This explanation negates the significance of finding that more controls possess only HSV-1 antibody.

Neither the patients and controls in the present study, nor in the study conducted by Skinner _et al_ (1977) were matched for sex related variables. This hypothesis can be tested accurately only by matching controls for sex related variables. It cannot otherwise be assumed that patients and controls are equally exposed to HSV-2. This is perhaps a generalisation which is more applicable to HSV-1.

Thus although the findings in this study confirm previous findings, it cannot be concluded that previous infection by HSV-1 protects from subsequent HSV-2 infection.

B. Previous HSV-1 infection protects from malignant transformation by HSV-2.

Of eighty-six British, Sri Lankan and Malawian patients with cervical anaplasia in this study who possessed antibody to herpes simplex virus, four (4.6%) possessed only HSV-2 specific antibody. The expected number was 3.7. This study therefore provides no evidence to support this hypothesis. Preliminary data from a prospective study conducted by Nahmias et al (1978) on five hundred ninety-six women with genital herpes said they had identified twenty-five patients who showed serological evidence of primary HSV-2 infection, i.e. these patients showed no evidence of previous HSV-1 infection. Cervical anaplasia developed in seven (28%) of the twenty-five patients with primary genital herpes and 14.6% in the total group of five hundred ninety-six patients. The authors however admit that the serological methods used to demonstrate HSV-2 infection may have been imprecise and state their intention to retest sera for HSV-1 and HSV-2 specific antibody by ELISA. If HSV-1 antibody is detected in these sera, their findings will be negated. A similar result was encountered in this study. Fifty-eight sera were independently tested by CF and ELISA. In three of fifty-eight only HSV-2 specific antibody was detected by CF, but in all three HSV-1 specific antibody also was detected by ELISA.

HERPES SIMPLEX VACCINES

Prevention of Marek's disease by vaccination with Turkey herpes virus vaccine has focused attention on the possibility of preventing other herpes virus related diseases by vaccination. The different types of vaccines which have been successfully used in Marek's disease are a) an avirulent Turkey herpes virus, b) an attenuated live Marek's disease virus, and c) a viral capsid vaccine (Purchase, 1976).

The suggestion has been made by Epstein (1976) that Epstein-Barr related diseases may be prevented by vaccination, and by Melnick (1976) and Skinner et al (1978) that cervical carcinoma may be prevented by a herpes simplex vaccine. A pre-requisite for such a human vaccine would be that it should be free of viral nucleic acid, and therefore of potential oncogenes.

Two types of herpes simplex vaccines have been prepared: a heat-inactivated HSV-1 and HSV-2 vaccines developed in Germany (Nasemann and Wassilew, 1979), and a virus particle-free vaccine developed in Birmingham, prepared by Nonidet NP-40 and formalin treatment of HSV-1 infected cell extracts (Skinner et al, 1978).

Clinical trials have been conducted with the German vaccine and the authors claim that the vaccine has been of value in the control of recurrent genital herpes. However the results reported are not very convincing. Of eleven patients who received vaccine, there was no clinical improvement in four, one patient had no further recurrences, and in

six the periods between recurrences were longer than they were prior to the administration of the vaccine and the attacks themselves were of shorter duration than before. The period of follow up of these patients was inadequate since it extended to only six months. This vaccine carries the added disadvantage of not being free of viral nucleic acid.

The vaccine developed by the Birmingham workers is free of viral DNA. It has not yet been used in a clinical trial. However, its protective effect has been established, for when administered intraperitoneally to mice which were later challenged by intravaginally introduced HSV-2, they were protected. The protective effect of the vaccine diminished when the challenge dose of virus was increased from 10^6 p.f.u. to 10^7 p.f.u., but the authors argue that penile lesions in humans usually yield titres of virus around 10^1 to 10^4 p.f.u. It is hoped by workers in the field, that herpes simplex vaccines would have two potential uses. Firstly, in the prevention or the modification of recurrent genital herpes and secondly in the prevention of cervical carcinoma.

It is not yet known whether cervical anaplasia is more likely to result from recurrent attacks of genital herpes rather than a single attack. If it is shown that recurrent attacks carry a higher risk of cervical anaplasia, and a vaccine can prevent such attacks, then a vaccine would be useful in the prevention of some cases of cervical carcinoma.

Another unknown factor which requires study is the possibility that different strains of HSV-2 may be of varying oncogenic potential. If such strains exist, it may be preferable to use them in vaccines prepared for the prevention of cervical

carcinoma provided of course that they are free of viral nucleic acid. On the other hand, workers such as Skinner et al (1977) have suggested that previous HSV-1 infection may protect, implying that HSV-1 vaccines should be used. In this context, animal experiments conducted by Duff, Dollar and Rapp (1973) sound a note of caution. Newborn Syrian hamsters were immunised with HSV-1 and HSV-2 prior to challenge with HSV-2 transformed cells. The animals formed neutralising antibodies to HSV but did not display any degree of transplantation immunity when compared with control animals injected with SV-40 and medium 199 respectively. Also surprisingly tumor metastases were significantly enhanced in HSV-1 immunised animals and suppressed in SV-40 immunised animals.

These experiments point to the possibility that HSV-1 may enhance the oncogenic effect of HSV-2. Could this be the reason why the majority of women with cervical anaplasia in the studies reported in this thesis as well as in other studies, possess HSV-1 as well as HSV-2 specific antibodies? Undoubtedly, many points need to be clarified before it would seem worthwhile or ethical to conduct vaccine trials on humans.

PREVIOUS INVESTIGATIONS ON ANTIBODIES TO MEMBRANE ANTIGENS OF HSV-2 INFECTED CELLS IN PATIENTS WITH CERVICAL ANAPLASIA.

Smith et al (1972b) examined the prevalence of these antibodies in thirty patients with invasive carcinoma and in thirty controls. They used an indirect immunofluorescent test employing goat anti-human globulin conjugate which was not class specific. The predominant class of antibody detected in this assay would therefore have been IgG. Neither the prevalence of membrane antibodies to HSV-2 infected cells nor their titres distinguished patients with invasive carcinoma from controls, these results being similar to IgG anti-MA results presented in this thesis.

Two studies showed complement dependent cytotoxic antibodies to HSV-2 infected cells to be of good prognostic value in patients with cervical anaplasia. However different results were obtained on assaying these antibodies to cell lines established from cervical carcinoma tissue. In the study by Thiry et al (1974) patients with cervical anaplasia were subdivided into two groups. There were eighteen patients with dysplasia, carcinoma in situ and stage III and IV invasive carcinoma with progressing lesions, and sixty patients with normal cervical smears who had previously had dysplasia or been treated for carcinoma in situ or invasive carcinoma, i.e. they were classified as pateints with regressing lesions. There was a higher prevalence (90%) of cytotoxic antibodies in patients with regresssing lesions than those with progressing lesions (56%). Similarly Christenson (1977) detected higher complement dependent cytolytic activity to HSV-2 infected cells in forty-three

patients who had been successfully treated for invasive carcinoma than in twenty-three terminal patients. Cytolysis was assayed by staining cells with trypan blue. She next assayed the same sera against cell lines established from cervical carcinoma tumor tissue. In contrast to results obtained employing HSV-2 infected cells, higher cytolytic activity to cervical carcinoma cell lines was detected in sera from terminal cases than survivors. This finding is interesting. It is possible with increase of tumor burden that there is antigenic competition between tumor specific antigen and HSV-2 antigen explaining the higher cytolytic activity to cervical carcinoma cell lines in terminal cases. During treatment by radiotherapy any virus or viral antigens in the cervical epithelium is likely to have been destroyed. After treatment there would be no tumor specific antigen to offer antigenic competition. Thus the increased cytolytic activity to HSV-2 infected cells observed in survivors is suggestive of reinfection of cervical epithelium by virus lying in neuronal ganglia. This study could have been better designed had these antibodies been assayed in serial samples of serum collected before and after treatment. Nevertheless they lend some support to suggestions put forward in this thesis to explain the persistence of IgA anti-MA following treatment of patients with cervical carcinoma (page 292).

Christenson and Espmark (1976) studied antibody to membrane antigens of HSV-2 infected cells by mixed haemadsorbtion in eighty-eight patients with cervical carcinoma, eighty-five controls, and seventy patients with extra-genital non-squamous malignancies. Seventy-one per cent of patients with cervical

carcinoma possessed these antibodies in contrast with 27% of controls and 27% of patients with extra-genital non-squamous malignancies. This distinction between patients with cervical carcinoma and the other two groups in the study is very similar to that obtained between similar groups employing the IgA anti-MA assay in the studies reported in this thesis (Fig. 6.2).

MARKERS OF PROGNOSTIC VALUE IN CERVICAL CARCINOMA

One of the aims of this study was to determine the prognostic value of monitoring HSV-2 related antibodies, serum immunoglobulins (IgG and IgA), and serum carcinoembryonic antigen (CEA) in the serial serum samples of patients with invasive carcinoma. The first question which arises is whether there is a need for such markers of prognosis. In answer to this question, a brief account is given below of the development of treatment regimes in cervical carcinoma including the presently used criteria for daignosis of tumor recurrences.

Until the end of the 19th century, once cervical carcinoma was diagnosed the maximum expectation of life was usually not more than two years (Stallworthy and Wiernik, 1976). The terminal stages of pateints with this malignancy were most distressing to both the patient and her family, for such complications as severe haemorrhages, and incontinence due to fistulae formation were common. Intractable pain associated with cachexia were inevitable terminal events. Despite advances in management, patients with recurrences still experience a similar terminal syndrome. Although the majority of deaths are currently seen in the developing countries there are also many women who die of the same syndrome in developed countries.

The first method of treatment for carcinoma of the cervix, was surgical, and fulfilled the principal laid down by John Hunter in the mid 19th century that "In destroying locally any specific disease which has power of its own increase the whole must be destroyed." Wertheim in 1898 per-

formed the first operation which has given him a place of honour in surgical history. By a strange coincidence, Marie and Pierre Curie isolated radioactive elements from pitch-blende in this same year and by 1903 radium was first used in the treatment of cervical carcinoma.

By 1930-1940 radiotherapeutic centres were established in many countries and the majority of women in the developed countries were treated for cervical carcinoma by radiotherapy. Many authorities such as Professor Heyman in Stockholm actively opposed the surgical treatment of cervical carcinoma. Even so, up to 60% of patients . . died of the terminal syndrome following recurrences of their tumors. The basic question which then arose was whether recurrent cervical carcinoma was caused by a new tumor growing in epithelium which had already demonstrated malignant potential or whether it arose from continuing growth of the carcinoma which was originally not completely destroyed by radiotherapy. The answer to this question was provided by Stallworthy who commenced his studies in 1939 although publication was delayed until 1964 (Stallworthy, 1964). He showed that recurrences developed from continuing growth of the carcinoma which was not originally destroyed by radiotherapy. From these observations developed the Oxford regime of treatment, which consists of radiotherapy followed in three months by a Wertheim's hysterectomy, the aim of which is to remove residual malignant cells. However, this regime is not followed by all gynaecologists. Some administer only radiotherapy, others perform only a Wertheim's hysterectomy. Some follow a Wertheim's hysterectomy by irradiation of the lymph nodes. All Sri Lankan patients in

study and the majority of British patients were treated by radiotherapy alone. Two British patients had Wertheim's hysterectomy and one had radiotherapy prior to Wertheim's hysterectomy.

Following therapy, suspicion of tumor recurrence is aroused only on such clinical grounds as a history of blood stained discharge, ulceration of the cervix or the detection of enlarged lymph nodes. Recurrences need to develop appreciably before any of these symptoms or signs appear.

A mark of early tumor recurrence would be useful in patients in stage I or II of the disease who have only been treated with radiotherapy for in such patients there is a place for performing rescue surgery in the event of tumor recurrence. Such a measure would be more successful if the recurrence can be detected before it is clinically apparent. It would also be helpful in cases where difficulties arise in distinguishing between tumor recurrences and non-malignant disease.

FINDINGS IN THIS STUDY

A change in level of immunoglobulins greater than or equal to 10% was considered to be a rise or fall in level as this is the normal inter-test variation observed in assays for total immunoglobulin by single radial immunodiffusion (Hobbs, 1970). Changes in titre of IgG anti-MA, IgA anti-MA and of total IgG and IgA were assessed at two stages during follow up. Firstly, at the end of ten weeks from the onset of treatment and secondly up to the end of the period of follow up. The usual course of radiotherapy lasts seven to eight weeks. Thus ten weeks was chosen as a time limit when it may have been expected that antibody titres and immunoglobulin levels would be stabilised following any disturbance caused by radiotherapy. Changes in level of CEA were assessed only at the end of the follow up period.

More patients who later developed recurrences of their tumor showed a rise in level of total IgG and IgG anti-MA at the end of ten weeks from the onset of treatment. However,

this difference was not as clear cut as the changes observed at the end of the period of follow up. By the end of the period of follow up a rise in level of total IgG was observed in five of five (100%) of patients who developed recurrences in comparison with five of fifteen (33%) of patients who remained well ($P < .01$). A rise in level of total IgA was observed in four of five (80%) patients who later developed recurrences in contrast with six of fifteen (40%) who remained well ($P > .05$) and a rise in titre of IgG anti-MA was observed in four of five (80%) patients who developed recurrences in contrast with five of fifteen (33%) who remained well ($P < .05$). Changes in IgA anti-MA and CEA did not provide a useful distinction between the two groups of patients.

Perhaps the findings in this study would have been more valid had the normal changes in immunoglobulin levels been assessed in serial samples from controls. The inter-test variation could also have been re-established. In this manner a more accurate estimate could have been made of what constituted a true rise or fall in level instead of the change of 10% referred to above. This could be done in future studies.

The suggestion made by Herbermann (1977) that in the immunediagnosis of malignancy, several tests used concurrently may provide more discriminatory data than a single test was extended in this study to the detection of tumor recurrences. Therefore the usefulness of all possible combinations of the three tests which showed promise as markers of tumor recurrence was evaluated. All combinations gave a greater degree of difference between the two groups of patients than any one test

did (Table 6.47B), but a rise in IgG anti-MA and a rise in total IgG provided the best distinction. Thus four of five (80%) patients who developed recurrences showed this combination of changes in contrast to one of fifteen (6%) who remained well.

Although the numbers of patients in this study are rather too small for firm conclusions to be drawn, these results have sufficient potential value to justify a more detailed study involving larger numbers of patients and controls. Nevertheless, the changes observed cannot be attributed to variation in treatment. Radiotherapeutic treatment administered to the two groups of patients at St. Thomas' Hospital was similar. None of the patients were given immunosuppressive drugs. It is possible that tumor recurrences make patients more susceptible to chronic cervical infection and this may account in part for the rise in total IgG. Whatever the reason for this phenomenon, if further studies confirm that it occurs consistently it would still serve as a useful marker of tumor recurrence. In this respect it was encouraging that changes in immunoglobulin and membrane antibody in Sri Lankan patients were similar to those observed among British patients.

Since serial serum samples from patients with invasive carcinoma were not collected on a monthly basis, it was not possible to ascertain the exact timing of these changes in relation to the clinical detection of recurrences, i.e. it is not known if these changes occurred before the recurrence was clinically apparent.

This study did not confirm the value of CEA in the detection of recurrent cervical carcinoma as shown by van Nagell et al (1978) (page 103). The reason for this may be that a different anti-CEA serum was used in the CEA assay employed in this study. In the study by van Nagell et al, Roche G-23 anti-CEA serum was used which recognises an ion sensitive site on the CEA molecule. However, considering the increasing evidence which shows the prognostic value of serial estimations of CEA in gastric cancer (Freeman et al, 1979) and colorectal cancer (reviewed in Neville and Cooper, 1976) it would be of interest to reassess levels of CEA in these sera employing the exact method employed by van Nagell et al.

As a marker of tumor recurrence, the non-specific nature of immunoglobulins may be advantageous. If cervical carcinoma is of multiple aetiology, it is likely that all agents would act via a final common pathway in bringing about malignant change. The final immune response to the tumor despite its aetiology might thus be expected to be the same. The same argument is applicable to CEA.

HSV-2 related antibodies which have been acclaimed as markers of prognostic value in cervical carcinoma are antibodies to AG-4. These antibodies were detected in a high proportion of untreated patients, but not in patients who had been successfully treated. They were also detected in patients who developed recurrence of their tumor (Aurelian et al, 1973, 1974b, and 1975). Although these results showed great promise, these workers have up to date not published further studies which have attempted to monitor these antibodies in serial samples of serum from a single group of patients followed

through from the time of initial diagnosis. Thus it is not known how long after therapy these antibodies became undetectable nor how long prior to clinical detection of tumor recurrence these antibodies reappear.

A COMPARISON OF THE COMPLEMENT FIXATION TEST (CF), ENZYME LINKED IMMUNOABSORBENT ASSAY (ELISA), AND MEMBRANE ANTIBODY BY FLUORESCENCE, AS ASSAYS FOR ESTIMATING PREVIOUS EXPOSURE TO HSV-1 AND HSV-2

Of all tests so far developed for estimating HSV-1 and HSV-2 specific antibodies in sera, only the CF test (Skinner et al, 1976) and ELISA (Grauballe and Vestergaard, 1977; and Vestergaard et al, 1977) employ type specific antigens. The type specific antibody of fifty-eight sera in this study were estimated by both these methods. In addition these sera were tested for IgG and IgA antibodies to the membrane antigens of HSV-2 infected cells. The sensitivity and usefulness of each test was evaluated by analysing the results obtained on each serum by all these assays.

Both the CF test and ELISA gave more false seronegatives (19% and 10% respectively) than the IgG anti-MA assay (3%). Thus as a method of determining previous herpes simplex infection (HSV-1 or HSV-2), the IgG anti-MA assay was the most sensitive. ELISA was the most sensitive in detecting HSV-1 specific antibody. It gave a false negative rate of 3.7% in contrast with a false negative rate by CF of 43%. ELISA was also the most sensitive assay for HSV-2 specific antibody, giving a false negative rate of 17% in comparison with a false negative rate of 34% by CF.

When the CF test for HSV type specific antibody was originally published, its efficacy in demonstrating type specific antibody in sera of immunised rabbits and in human

sera was described (Skinner et al, 1976). The test was more sensitive in detecting type specific antibody in convalescent sera than in sera collected long after initial infection. Thus HSV-2 specific antibody was detected in all human convalescent sera from HSV-2 infected patients. However, concurrent testing by neutralisation test and CF revealed that in the sera of HSV-1 seropositive nuns there was a 20% false negative rate for HSV-1 specific antibody by CF. Thus the higher rate of false negatives by the CF test in comparison with ELISA, may be a reflection that complement fixing antibodies in herpes simplex infection are shorter lasting than non-complement fixing antibodies. A similar loss of complement fixing-antibodies is seen in other viral infections such as influenza, measles and rubella. Following cytomegalovirus and EBV infections complement fixing antibodies may persist for a long duration of time and these may be due to the endogenous presence of virus with periodic recrudescence. A parallel situation may exist in herpes simplex infection, with persistence of complement fixing antibodies only in subjects harbouring latent herpes virus and who experience recrudescences. Another contributory factor to the high rate of false negatives by CF may be the sensitivity of the CF test itself. According to Voller et al (1976) ELISA may be of a sensitivity comparable with radioimmunoassay.

From studies conducted by Skinner et al (1976) and the present study, there is no doubt that antigen employed in the CF test is type specific. Perhaps more profitable use may be made of these laboriously prepared antigens by employing them in a sensitive assay such as ELISA or radioimmunoassay

for non-complement fixing antibodies.

The IgA response to HSV-1 and HSV-2 infection was found to be more type specific than the IgG response. This may be put to clinical use in determining infecting virus type in the event of failure to isolate virus. It would be of interest to determine if such IgA antibodies may be detected in the cerebrospinal fluids of patients with herpes encephalitis.

On examining IgA anti-MA titres of forty-three British sera which had previously been tested by ELISA, it was found that a titre of greater than or equal to 1:8 for IgA anti-MA indicated the presence of HSV-2 specific antibody and titres of less than 1:8 its absence. As a method of estimating the presence of HSV-2 specific antibody this dividing titre of 1:8 gave a false result rate of 20% among British subjects and 25% among Sri Lankan subjects. Among Malawian subjects the corresponding titre was 1:16 with a false result rate of 17% (both false positive and false negative). This false result rate of 17-25% compares very favourably with corresponding false result rates by CF (34%) and ELISA (17%). Thus while the IgG membrane antibody assay on HSV infected cells provides a very sensitive method of determining type common herpes simplex antibody, concurrent testing of sera by the IgA membrane antibody assay would be of value in determing which type specific antibody was present. However, it does not differentiate between sera containing HSV-2 specific antibody alone and sera which contain both HSV-1 and HSV-2 specific antibody. Nevertheless this is not a great disadvantage for only five sera among all those tested in this study had HSV-2 specific antibody alone.

The higher dividing titre of 1:16 observed among Malawian subjects is in keeping with the geographic variation in titre of IgG anti-MA and IgA anti-MA that was observed in this study. Malawian subjects possessed higher titres than subjects from other countries. This geographic variation may have resulted from an inter-country variation in the background prevalence of HSV-1 infection. It may also be a reflection of the influence of such environmental factors as parasitic infections which may alter the immune reactions to certain antigens (McFarlane, 1973).

The dividing titre of 1:16 also provides an explanation for the fact that similar proportions of Malawian patients and controls possessed IgA anti-MA greater than 1:4 (Table 6.2). In contrast, a significantly higher proportion (66%) of Malawian patients with invasive carcinoma had IgA anti-MA greater than 1:16 when compared with controls (38%) ($P < .05$).

CONCLUSIONS

CONCLUSIONS

1. STUDIES RELEVANT TO SCREENING FOR CERVICAL CANCER

It was found that the cervical screening programme which has been in operation for over ten years in the U.K. has made no impact on incidence rates or mortality rates from the tumor. Some possible contributory factors were identified in this study. Firstly, although the incidence of cervical anaplasia in women below thirty-five years is rising, the present screening policy is to perform routine cervical smears only on women above this age. This study provided evidence which supported recent suggestions made that this age should be reduced. Secondly, data obtained from this study suggested that the screening programme was not being sufficiently promoted among women in the lower socio-economic groups and among post-menopausal women.

There is a need to screen by Papanicolaou smear, Malawian, Sudanese, and Sri Lankan women, as they have an early age specific incidence of the tumor. As cervical carcinoma was found to occur well within the child bearing age in these countries, screening could be conducted at antenatal clinics.

2. STUDIES ON THE EPIDEMIOLOGICAL ASPECTS OF CERVICAL CARCINOMA

This study confirmed previous findings that early initiation of coitus was an epidemiological feature of patients with cervical anaplasia among patients in all four countries in this study. It is therefore concluded that the relationship between early initiation of coitus, genital herpes and

cervical anaplasia requires further clarification before HSV-2 could be accepted as being a causal agent of cervical anaplasia.

3. STUDIES ON ANTIBODIES TO THE MEMBRANE ANTIGENS OF HSV-2 INFECTED CELLS

The IgA response to the membrane antigens (MA) of HSV-2 infected cells was found to be more type specific than the IgG response. This finding has potential clinical value.

In the absence of clinical herpetic infection the hypothesis was put forward that IgA anti-MA resulted from the expression of early viral proteins by latent HSV-2 virus which lay at sites outside the nervous system. A high prevalence of IgA anti-MA was detected in patients with cervical anaplasia in titres similar to those observed in convalescent sera of patients with genital herpes. It was therefore suggested that a high proportion of these patients have not only been previously infected with HSV-2 but may continue to harbour latent virus.

The high prevalence of IgA anti-MA in patients with non-genital squamous carcinoma was explained by evidence suggestive of a cross reacting antigen between HSV-2 infected cells and squamous carcinoma cells. Antibodies to this antigen were detected by the IgA membrane fluorescence assay. They were in part contributory towards the high titres of IgA anti-MA in patients with carcinoma <u>in situ</u> and invasive carcinoma, and to the rise in titre of IgA anti-MA with increase with the stage of disease. It was concluded that this preliminary

evidence for a cross reacting antigen deserved further investigation as antibody to it in patients with cervical carcinoma could contribute towards a false result of elevated antibody titres to HSV antigen.

4. STUDIES ON THE PREVALENCE OF HSV-2 SPECIFIC ANTIBODIES (ELISA) IN PATIENTS WITH CERVICAL ANAPLASIA

HSV-2 was associated with cervical anaplasia in Britain, Sri Lanka and Malawi since patients with cervical anaplasia had a significantly higher prevalence of HSV-2 specific antibody when compared with controls. However the prevalence of HSV-2 specific antibody in cohorts of British patients and controls suggested that the virus may have a role as a co-variable of the real carcinogen rather than be the causal agent itself. Even its role as a co-variable was not universal since up to 48% with cervical carcinoma in the Sudan did not show evidence of previous infection by HSV-2. Among patients with cervical anaplasia from Malawi and Britain this lack of HSV-2 specific antibody was more apparent in women who developed cervical carcinoma at an older age.

5. STUDIES ON THE PREVALENCE OF HSV-2 SPECIFIC ANTIBODY (ELISA) AND IgA ANTIBODY TO THE MEMBRANE ANTIGENS OF HSV-2 INFECTED CELLS IN PATIENTS WITH MALIGNANCIES OTHER THAN CERVICAL CARCINOMA

There was insufficient evidence to support that HSV-2 was associated with extra-genital non-squamous malignancies and with other genital malignancies, but non-genital squamous malignancies were nearly as strongly associated with HSV-2

as cervical anaplasia. Antibody to a cross reacting antigen between HSV-2 infected cells and squamous carcinoma cells may have contributed to this phenomenon. Firm conclusions cannot be drawn until sera from a larger number of patients with non-genital squamous carcinomas are examined and attempts made to characterise this cross reacting antigen. It is therefore concluded that patients with extra-genital squamous malignancies should form an important control group in future studies on the nature of the association between HSV-2 and cervical carcinoma.

6. STUDIES ON THE PROSPECT OF PREVENTING GENITAL HERPES AND/OR CERVICAL CARCINOMA BY THE USE OF HERPES SIMPLEX VACCINES

There was no evidence from this study to show that previous HSV-1 infection protected from subsequent HSV-2 infection or from developing cervical carcinoma.

7. STUDIES ON MARKERS OF PROGNOSTIC VALUE IN CERVICAL CARCINOMA

Rising levels of total IgG, total IgA and IgG anti-MA (HSV-2) were associated with tumor recurrences. A combination of a rise in total IgG and IgG anti-MA were the best markers of tumor recurrences. However this finding needs to be confirmed in larger numbers of patients. Investigations also need to be carried out as to the exact timing of these changes in relation to occult tumor recurrences in order to assess the clinical value of these serological markers.

8. STUDIES COMPARING THE SENSITIVITY OF ENZYME LINKED IMMUNOABSORBENT ASSAY (ELISA), COMPLEMENT FIXATION (CF) AND MEMBRANE FLUORESCENCE FOR DETECTING PREVIOUS INFECTION BY HSV-1 AND HSV-2

The IgG membrane fluorescence assay was the most sensitive in detecting type common antibodies. The ELISA technique was the most sensitive in detecting HSV-1 and HSV-2 specific antibodies. The higher false negative rate for type common antibody and HSV-1 and HSV-2 specific antibody by the CF test may be an indication that in contrast with non-complement dependent antibodies complement dependent antibodies in herpes simplex infection are time dependent.

The IgA membrane antibody assay was useful in determining the presence or absence of HSV-2 specific antibody.

Chapter 8

FUTURE STUDIES

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FUTURE STUDIES

STUDIES THAT WOULD CLARIFY THE RELATIONSHIP BETWEEN EARLY INITIATION OF COITUS, CERVICAL ANAPLASIA, AND GENITAL HERPES, AND THE ROLE PLAYED BY OTHER SEXUALLY TRANSMITTED AGENTS.

A prospective study on patients presenting at different ages with their first attack of genital herpes with cervical involvement would help to clarify this question. It would be necessary to conduct regular colposcopic examination of their cervices, e.g. once in three to six months. Abnormal colposcopic findings should be confirmed by Pap smears and/or biopsies. Such a study should aim to establish whether colposcopically abnormal transformation zones develop more frequently following genital herpes infection during adolescence, or following first pregnancy than at other times. In addition it could be established whether a single attack of genital herpes or recurrent attacks have the same or different effects on the cervical epithelium. The follow up period should be long in order to assess whether abnormal lesions which develop are reversible or persistent. A group of women with abnormal transformation zones who do not give a previous history of genital herpes could form a suitable control group, and may be picked out at colposcopy clinics. Antibodies to HSV-2, mycoplasma, chlamydiae, gonococci, cytomegalovirus and human papilloma should be monitored in the sera collected at each visit for colposcopic examination. Thus the role of many sexually transmitted agents may be studied. A history relating to ethnic origin, social class, and sex related variables would be necessary for the meaningful analysis of results.

STUDIES DESIGNED TO ESTABLISH THE SITE OF LATENCY OF HSV-2

If there is latent virus in cervical epithelium it is likely that HSV-2 specific antibody would be found in cervical mucus. It is usually not possible to collect cervical mucus from patients with cervical carcinoma. Thus cervical mucus and cervical epithelial tissue from women with healthy cervices, with a previous history of a single attack of genital herpes, with recurrent attacks of genital herpes, with carcinoma *in situ* and with dysplasia could be used. Levels of total immunoglobulin and IgG and IgA antibodies to the membrane antigens of HSV-2 infected cells could be examined in cervical mucus specimens.

Exfoliated cells and preferably cryostat sections of biopsy materials could be examined for herpes antigens and herpes DNA. Anti-HSV sera raised in animals, or monospecific sera raised in animals to HSV glycoproteins could be used in either a fluorescent test or a peroxidase label test in the study of herpes antigens. The methods of *in situ* hybridisation used by McDougal (page 314) could be used to detect herpes DNA. Since in this study there was evidence for a cross reacting antigen between HSV-2 infected cells and squamous carcinoma cells, biopsy material from squamous carcinomas would form important control material in the herpes antigen studies.

If biopsy material is collected, it would be relevent to determine the class of antibody and the herpes specificity of antibodies in plasma cells in the cervical epithelium.

In countries where Wertheim's hysterectomy is performed without prior radiotherapy, such investigations could be extended to studies on class specificity and herpes specificity of antibodies eluted from cancerous tissue.

STUDIES ON AN ANTIGEN COMMON TO HSV-2 INFECTED CELLS AND SQUAMOUS CARCINOMA CELLS

Antibodies to such an antigen appeared to be detected by the IgA membrane fluorescence assay used in the studies reported in this thesis. Thus IgG should be removed from the sera of patients with cervical carcinoma and non-genital squamous carcinoma which contained high IgA anti-MA titres. Such IgG free fractions could be tested by the intermediate gel technique of crossed immunoelectrophoresis described by Vestergaard et al (1979) against solubilised HSV-2 infected cells and squamous carcinoma cells. IgG free fractions of the following control sera should also be used in the study:

1. IgA anti-MA positive sera from patients with genital herpes, dysplasia and from women with healthy cervices.
2. IgA anti-MA negative sera.

Depending on the results of the above studies, methods could be devised to characterise this cross reacting antigen further.

STUDIES TO EXAMINE THE POSSIBILITY THAT THE AETIOLOGY OF CERVICAL CARCINOMA SHOWS GEOGRAPHIC VARIATION AND VARIES ACCORDING TO AGE.

Sera from two or more contrasting epidemiological groups could be examined, e.g. patients with invasive carcinoma and controls from:

1. a country where an association of the tumor with HSV-2 has been established,
2. a country where no such association has been established, and
3. an epidemiological group which has a low incidence of the tumor.

Controls should be matched for ethnic origin, sex, age, social clas and sex related variables.

In addition sera should be collected in each country from patients with non-genital squamous carcinomas.

Knowledge of the pattern of sexually transmitted disease in the environment from which each sample is drawn would be necessary for analysis of such data.

It would be possible in such a study to draw conclusions from an inverted case control prevalence of HSV-1 antibody (see page 317).

IN VITRO STUDIES WHICH MAY INDICATE WHETHER HSV-2 IS CAUSALLY RELATED TO CERVICAL CARCINOMA

Few transformation experiments have been conducted on human cells (see Table 1.5). Vesterinen et al(1975) have shown that it is possible to establish cell lines from squamous epithelium and columnar epithelium covering the cervix. Thus attempts could be made to transform with HSV-2, cell lines established from normal squamous epithelium. If such attempts are successful, the antigens on these transformed cells could be compared with those on cell lines in low passage which have been established from squamous cervical carcinoma biopsy material.

Control cell lines established from biopsy material from non-genital squamous carcinomas should also be used.

STUDIES WHICH MAY IDENTIFY FACTORS CONTRIBUTING TOWARDS THE FAILURE OF THE PRESENT SCREENING PROGRAMME IN THE U.K. TO REDUCE INCIDENCE RATES AND MORTALITY RATES FROM CERVICAL CARCINOMA

An assessment needs to be made of the proportion of those at risk who are being screened at present. Also an estimation according to regions of the proportion of invasive carcinomas being detected by smears and the proportion due to clinical symptoms, would identify areas where the programme is ineffective. Accurate information on the incidence of invasive carcinoma according to social class, ethnic origin and age on a regional basis would identify high risk groups which the screening programme has failed to reach adequately. An assessment should be made of the efficiency of the present system in ensuring re-call for follow up of women who have had abnormal smears.

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INFORMATION - PATIENTS

A. GENERAL INFORMATION

Name: CB No:

Age:

Ethnic origin:

Husband's occupation: Social class:

Patient's occupation:

Age at marriage:

Number of pregnancies:

B. DIAGNOSIS AT TIME OF COLLECTING FIRST SPECIMEN OF SERUM

Tick off what is applicable -

Dysplasia ☐

C_A in situ ☐

Invasive C_A ☐

C_A in situ reverted to normal after conisation ☐

Dysplasia reverted to normal (1) spontaneously ☐ (2) after conisation ☐

Treated invasive C_A no recurrence ☐

Treated invasive C_A showing recurrence ☐

C. CLINICAL STAGE OF NEOPLASM

Tick off what is applicable -

Stage I ☐

Stage II ☐

Stage III ☐

Stage IV ☐

D. PAPANICOLAOU STAIN REPORT

E. TREATMENT

Form of treatment - tick off what is applicable: Radiotherapy ☐

Hysterectomy ☐

Date treatment was instituted: _____

Date treatment was discontinued: _____

Date recurrence was noticed: _____

F. SPECIMENS

Date of collecting serum specimens:

1st specimen _____

2nd specimen _____

3rd specimen _____

4th specimen _____

5th specimen _____

INFORMATION - CONTROLS

Name: CB number:

Age: Address:

Ethnic origin:

Complaint/Diagnosis:

CIVIL STATUS

Married

Not married
- a) dependent on parents
- b) self-supporting
- c) living with boy-friend

Divorced

Separated

Number of children:

EMPLOYMENT STATUS

*Husband/boy-friend/father/self

Employed - job:
- a) manager
- b) foreman
- c) trained, self-employed
- d) trained, working for employer
- e) untrained

Unemployed } - previous job:
Retired }

Patient's occupation: Social class:

MEDICAL HISTORY

Has Pap-smear been done? Yes/No

Date

Place

Normal/Abnormal

Previous history of
- a) genital herpes
- b) non-genital herpes

*Cross out when not applicable.

Serum.....

APPENDIX B

EQUIPMENT AND MATERIALS REQUIRED FOR ASSAYING ANTIBODIES TO MEMBRANE ANTIGENS OF HERPES SIMPLEX INFECTED CELLS, AND VIRUS CAPSID ANTIGENS OF HSV-2 INFECTED CELLS.

1. Polystyrene bottles 150 cm^2	Costar
2. Microtitre plates U-shaped	Flow Laboratories
3. Disposable pipettes 0.025 ml (cat. no. 77-020-06) 0.05 ml (cat. no. 77-021-06)	Titertek Microtitration equipment . Flow Laboratories.
4. Finn Pipette 5 µl - 50 µl	Jencons Scientific Ltd.
5. Disposable tips	Alpha Laboratories.
6. Hand Multidiluter (cat. no. 77-100-00)	Titerek Flow Laboratories.
7. Microscope slides 76 x 25 mm	C.E. Payne and Sons Ltd.
8. Multispot Microscope Slides	C.A. Hendley and Company.
9. Round Cover Slips 13 mm diameter No. 0	Chance Propper Ltd.
10. Rectangular Glass cover slips 22 x 64 mm No. 1½	Chance Propper Ltd.
11. Rotor Mixer de Luxe	Hook and Tucker Ltd.
12. Shaker	Luckham Ltd.

13. Centrifuge
    Mistral 4L — MSA Scientific Instruments

14. Fluorescent Microscope
    with epifluorescence : condenser — Zeiss

15. Ultrasonic disintegrator
    PG 100, Model 150 W. — MSE Scientific Instruments

16. Heat Sealable Ampoules
    2 ml (cat. No. 508) — Sterilin Ltd.

17. Rabbit Kidney Cells (RK 13)

RK 13 cell line (Glaxo RK 13) was obtained from the Medical Research Council Laboratories. The cells used in this study were all below the 40th passage. The cells were grown till the cell sheet was confluent (3rd day usually) and left on maintenance medium for 2 days prior to splitting. The cells were split one to three using a trypsin and versene solution.

Storage of cells

Cells from one 150 cm^2 bottle were suspended in 3 ml of minimal essential medium containing 20% Fetal Calf Serum (B20) and 10% Dimethylesulphoxide (B29). This was aliquoted in one ml. quantities in heat sealable ampoules (B16), frozen in a liquid nitrogen cylinder and stored in a gas phase nitrogen cylinder.

Seeding of cells

The cells in each ampoule were thawed at 37°C, washed once in growth medium, centrifuged at 1000 x g for 10 minutes in a bench centrifuge, and resuspended in 50 ml of growth medium in a 150 cm^2 bottle.

18. Growth Medium

Minimal Essential Medium Eagle (Modified) (MEM)
with Earl's salts
with 0.85 g/l sodium bicarbonate — Flow Laboratories

5% Fetal calf serum
1% Glutamine
1.3% of a 4.4% sodium bicarbonate solution
2% Penicillin and Streptomycin solution

19. Maintenance Medium

MEM as above
2% Fetal Calf serum
1% Glutamine
2.4% of a 4.4% solution of sodium bicarbonate
2% Penicillin and Streptomycin solution

20. Fetal Calf Serum

(Cat. No. 29-101-54) Flow Laboratories

21. Glutamine

Sterile 200 mM L-Glutamine (Cat. No. 16-801-48)
Flow Laboratories

22. 4.4% Sodium Bicarbonate

44 gms sodium bicarbonate
100 ml distilled water
1 ml 1% phenol red
The bicarbonate solution was prepared using the materials above. Dry ice was added to saturate the solution with carbonic acid and 25 ml aliquots were sterilized by autoclave.

23. Trypsin and versene solution

a) 1 gm of versene (EDTA-diaminoethane tetra acetic acid disodium salt) BDH Chemicals Ltd.
This was dissolved in 2 L of PBS (B25). The solution was autoclaved in aliquots of 100 ml and stored at 4°C.

b) Sterile freeze-dried 5% trypsin

Wellcome Laboratories
One vial of trypsin was reconstituted in 10 ml of versene solution and made up to 200 ml with more versene solution. This gave a dilution of 0.05% versene and 0.25% trypsin. This solution was aliquoted and stored at -20°C.

24. Penicillin and Streptomycin Solution

Penicillin one ampoule (1 million units)
Streptomycin one ampoule (1 million g)
100 ml sterile distilled water.
Aliquots of 12 ml were stored at -20°C (penicillin 10,000 units/ml and streptomycin 10,000 g/ml).

25. Phosphate buffered saline (PBS)

PBS, Dulbecco 'A' was prepared by dissolving one tablet (Oxoid Ltd.) in 100 ml of deionized water, sterilized by autoclaving for 15 minutes at 15 lbs pressure and stored at room temperature.

26. Veronal buffer (5x)

83 gms NaCl and 10.19 gms Na-5-5 diethyl barbiturate was dissolved in 1500 ml distilled water. 31.2 ml of N/I HCl was added to this. The buffer was made up to 2000 ml, and stored at $4^{o}C$.

Before use, a 5-fold dilution of the above 5x buffer was prepared by adding distilled water.

27. Glycerol

Analytic reagent BDH Chemicals Ltd.

28. Acetone

Analytic reagent BDH Chemicals Ltd.

29. DMSO (Dimethyl sulphoxide)

Stored at room temperature Hopkins and Williams.

30. Virus

Both strains of HSV used were provided by Dr. G.R.B. Skinner, of the Department of Virology, The Medical School, Birmingham, England.

HSV-1

Strains HF, the HFEM derivative of the Rockefeller strain HF (Wildy, 1955) was used.

HSV-2

BRY - an isolate from a 19 year old patient with recurrent genital herpes was used.

Both viruses had been passaged several times in tissue culture prior to use. They were employed as prototype HSV-1 and HSV-2 in the study on the differentiation of type 1 and type 2 strains of HSV by indirect immunofluorescent technique (Geder and Skinner, 1971).

Propagation of virus

Virus was grown in RK 13 cells which were inoculated on the 3rd or 4th day after splitting with 5 p.f.u. per cell. Virus was allowed to adsorb for one hour at room temperature after which the virus inoculum was discarded. 50 ml of maintenance medium was added and the cells incubated at 37°C for approximately 12 hours until a 75% CPE developed. The cells were shaken into the medium with the use of sterile glass beads. The medium containing the suspension of infected cells was aliquoted into 20 ml aliquots in universals and snap-frozen in dry ice and methanol. This was stored at -70°C. Prior to use, the cell suspension was sonicated for 10 seconds (16 at 20 Kc/sec).

31. Conjugate-Fluorescein conjugated animal antiglobulin

1. Rabbit antihuman IgG-Fluorescein isothiocyanate Batch No. 4-572
2. Rabbit antihuman IgA-Fluorescein isothiocyanate Batch IO-173

Nordic Pharmaceutical and Diagnostic.

APPENDIX C

Virus Titration

Eggs - Fertile eggs incubated at 100°F for 10-12 days.
Dentist's drill Citenco Elstree

Method

The eggs were candled, and using a carborundum disc fixed to the dentist's drill a perforation was made in the shell over the air sac and a triangular portion of the shell removed over the embryo. Care was taken that this triangular portion did not lie over blood vessels, and that the drilling process did not penetrate deeper than the shell. A light scratch was made on the shell membrane with a sterile injection needle and the chorioallantoic membrane (CAM) dropped by sucking with a large teat placed over the perforation over the air sac. The eggs were candled again to check that the CAM had dropped.

Virus dilutions

10-fold dilutions of the virus was made in sterile PBS in bijoux bottles in an ice bath.

Inoculation of virus onto CAMs

0.1 ml of each virus dilution was inoculated onto each CAM of 3 eggs. A 1 ml disposable syringe with a 25 gauge injection needle was used. 2 CAMs were inoculated with 0.1 ml of PBS. The eggs were gently rocked after inoculation to spread the inoculum on the CAM.

Incubation and the reading of CAMs

The performations on the shell were sealed with cellotape, and the eggs incubated at 37°C for 72 hours. The CAMs were harvested, washed, and those containing 20-50 pocks were used in estimating virus titre. The mean pock count on the three CAMs inoculated with that dilution of virus was used.

APPENDIX D

Method for production of pig liver powder

Materials

Pig's liver	
Thiomersal	BDH Laboratories
Acetone (B28)	
Homogeniser	
Centrifuge (B13)	
Whatman 54 filter paper	W and R Balston Ltd.

A fresh pig liver was obtained with the portal vessels intact. The liver was washed with tap water through the venous system until pale. It was next cut into pieces and washed with saline. The pieces were homogenised with 3 volumes of saline, and centrifuged at 2000 rpm for 45 minutes.
The sediment was resuspended in saline containing 0.01% thiomersal, and left at $4^{o}C$ overnight. The suspension was centrifuged at 2000 rpm for 30 minutes.
The sediment was resuspended in saline with 0.01% thiomersal and left overnight. The suspension was rehomogenised, and passed through a double layer of muslin. The filtrate was centrifuged at 2000 rpm for 45 minutes.
The sediment was resuspended in one litre of acetone and filtered through a funnel using Whatman 54 filter paper. The sediment was washed further with acetone until white. It was dried at $37^{o}C$ in the incubator, and ground to a fine powder in a homogeniser

APPENDIX E

Purification and treatment of conjugates prior to use

Freeze-dried IgG and IgA conjugate (Appendix B31) was reconstituted with sterile distilled water according to the manufacturer's instructions. It was absorbed with pig liver powder (PLP) (Appendix D) to purify it of unreacted fluorescent material.

Absorption with pig liver powder

100 mg of PLP per ml of reconstituted conjugate was used. The PLP was washed twice with distilled water and once in PBS prior to use. It was shaken gently with conjugate for one hour at $4^{o}C$. The mixture was centrifuged at 3000 rpm in a bench centrifuge and the purified conjugate aliquoted and stored at $-70^{o}C$.

Absorption with control cells

About 2-3 hours prior to use, a 1:2 dilution of the conjugate was prepared in PBS and absorbed with uninfected RK 13 cells. The cells used thus for absorbing may be harvested and stored at $-70^{o}C$ over long periods of time. Approximately 8×10^{6} cells (cells from one 75 cm^{2} bottle) were used per 0.4 ml of 1:2 dilution conjugate. Absorption was carried out with gentle shaking at $+4^{o}C$ for one hour. At the end of one hour, the mixture was centrifuged at 3000 rpm in a bench centrifuge and the conjugate was diluted with PBS to its use dilution.

APPENDIX F

Treatment of sera showing non-specific fluorescence

These sera were absorbed with control cells on the day they were being tested. A 1:2 dilution of serum was prepared in PBS by adding 0.2 ml of PBS to 0.2 ml of serum. This was gently shaken with approximately 8 x 10^6 RK 13 cells (cells harvested from one 75 cm^2 bottle) for one hour at 37°C. The serum cell suspension was centrifuged at 3000 rpm in a bench centrifuge for 5 minutes. Next the serum was absorbed with RK 13 cells of the same quantity as above, for one hour at 4°C. The cells were removed by centrifugation and the serum assayed immediately as the antibody titre of serum absorbed thus at a 1:2 dilution deteriorated on storing.

APPENDIX G

Materials and equipment required for serum IgG and IgA determination by single radial immunodiffusion

1) Accra assay IgG and IgA regular level single radial immunodiffusion plates.
   IgG - code no. 61-303-1
   IgA - code no. 61-301-1
   stored at 4°C in the inverted position
   Miles Laboratories

2) Accra assay reference human sera, high, mid-range, and low for both IgG and IgA.
   Stored at 4°C.

3. Micropipette
   capillary pipette delivering 10 microlitres

4) Diameter measuring template
   Behringwerke AG

5) X-ray viewer
   Wardray Products Ltd.

APPENDIX II

Equipment and materials required for Rubella and measles HAI tests.

1. Microtitre plates
   V-shaped (cat. no. 76-321-05) Flow Laboratories

2. Dilutors
   Linbro, 0.025 ml (cat. no. 76-742-00) Flow Laboratories

3. HAI diluent
   To 96 ml of veronal buffer, 2 ml of a 10% solution of bovine plasma albumen and 1 ml each of a 10% solution of calcium chloride and 8% magnesium sulphate solution were added. The mixture was shaken and the pH adjusted to 6-7 with sodium bicarbonate (0.044%).

4. Rubellar HAI antigen
   Cat. no. 40-809-41 Flow Laboratories

5. Measles HAI antigen
   Cat. no. ORAO 09 Behring Diagnostics

6. Veronal buffered saline (CFT diluent)
   This was prepared using CFT diluent tablets (Oxoid Ltd). Each tablet was dissolved in 100 ml deionized water.

Formula	g/litre
Barbitone	0.575
sodium chloride	8.500
magnesium chloride	0.168
calcium chloride	0.028
barbitone soluble	0.185

pH 7.2 (approx.)

7. Bovine plasma albumin

   A 10% solution of BPA powder (Armour Pharmaceutical Co. Ltd) was prepared in PBS 'A' (Appendix B25). The solution was sterilized by passing through a millipore filter and stored at 4°C.

APPENDIX J

Levels of total IgG and IgA and titres of IgG anti-MA and IgA anti-MA in patients with invasive carcinoma from Britain, Sri Lanka and Malawi were analysed by a multi-way analysis of variance to take into account a combination of factors such as race, age and stage of disease. The computer program GLIM was used as observations in the various groups were unequal. For the actual analysis, logariths of the measurements were used but results shown below show 95% confidence intervals for the mean ratios in actual measurements. This analysis was done by R.W. Morris of the Department of Community Medicine at St. Thomas' Hospital.

1) IgG anti-MA

Race was overall a significant factor when adjusted for other factors (i.e. stage of disease and age) ($P < 0.01$). The differences in estimated measurements for the various races are shown below.

If M-B = Ratio of Malawians to British
M-SL = " " " " Sri Lankans

$2.4756 \leq M\text{-}B \leq 11.9831$
Mean = 5.4466

$0.6422 \leq M\text{-}SL \leq 1.7393$
Mean = 1.2422

The other two factors (stage, age) were not significant.

2) IgA anti-MA

No significant results were found. In a model including all three factors, estimated measurements were highest for Malawians and lowest for British, but the overall difference was not significant. They were lowest for patients in stages 1 and 2, and highest for those in stage 4, suggesting some trend according to stage of disease: however this result is not significant.

3) Total IgG

In the usual model incorporating all three factors, the overall effect of race was found to be highly significant ($P < 0.01$); estimated measurements for British were lower than the other two races.

If SL-B = Ratio of Sri Lankans to British
M-B = " " Malawians " "

$1.412 \leq$ SL-B ≤ 2.1606
Mean = 1.7467

$1.2789 \leq$ M-B ≤ 2.1255
Mean = 1.6487

Estimated measurements increased according to stage but the overall difference was not significant.

If ST4-1 = Ratio of stage 4 patients to stage 1 patients
$1.0282 \leq$ ST4-1 ≤ 2.1797
Mean = 1.497

4) Total IgA

Estimated measurements were lower for Malawians than the other two races.

Let B-M = Ratio of British to Malawians
B-SL= " " " " Sri Lankans

$0.9833 \leq$ B-M ≤ 1.6441
Mean = 1.2715

and

$0.7326 \leq$ B-SL ≤ 1.1261
Mean = 0.9083

The overall test for the effect of 'race' gave a p-value of less than 0.1 but greater than 0.05.

The estimated measurements increased according to stage of disease, and this time the overall effect of 'stage' was significant ($P < 0.01$).

Let ST2-1 = Ratio of Stage 2 patients to Stage 1 patients
ST3-1 = " " " 3 " " " " " "
ST4-1 = " " " 4 " " " " " "

$0.9494 \leq$ ST2-1 ≤ 1.6408
Mean = 1.2422

$1.0160 \leq$ ST3-1 ≤ 1.8228
Mean = 1.361

$1.4989 \leq$ ST4-1 ≤ 3.3112
Mean = 2.2278

The older the patient, the lower the estimated measurement. This effect has a p-value of around 0.1.

If F = Factor by which the measurement is multiplied every year.

$$0.9990 \leqslant F \leqslant 1.0139$$

Mean = 1.0064

<u>5) IgA anti-MA</u> was once again analysed - using total IgA as another factor (covariate). So the model used to analyse specific IgA included age, race, stage and total IgA.

IgA anti-MA seemed to decrease with increasing total IgA.

When total IgA was doubled, specific IgA was multiplied by a factor F.

$$0.3682 \leqslant F \leqslant 1.1338$$

Mean = 0.6461

This effect, however, is not significant.

Acknowledgements

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This study relied on the collection and transport of a number of specimens from outside the U.K. I extend my gratitude to Professor M.S.R. Hutt of St. Thomas' Hospital and Dr. J. Chipangwi of the Queen Elizabeth Central Hospital at Blantyre, Malawi for specimens from Malawi; to Dr. M.O.A. Malik for specimens from Sudan; and for Sri Lankan specimens to Dr. Lorraine Senarath of the Cancer Institute, Dr. S. Thawarasa of the Colombo Medical Faculty, and Dr. C.L. Mendis of the Medical Research Institute.

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firming some of my statistical tests. My thanks are also due to Dr. K.E.K. Rawson of the Institute of Laryngology and Otology and Dr. K.J Randall at Orpington Hospital for additional sera from British patients.

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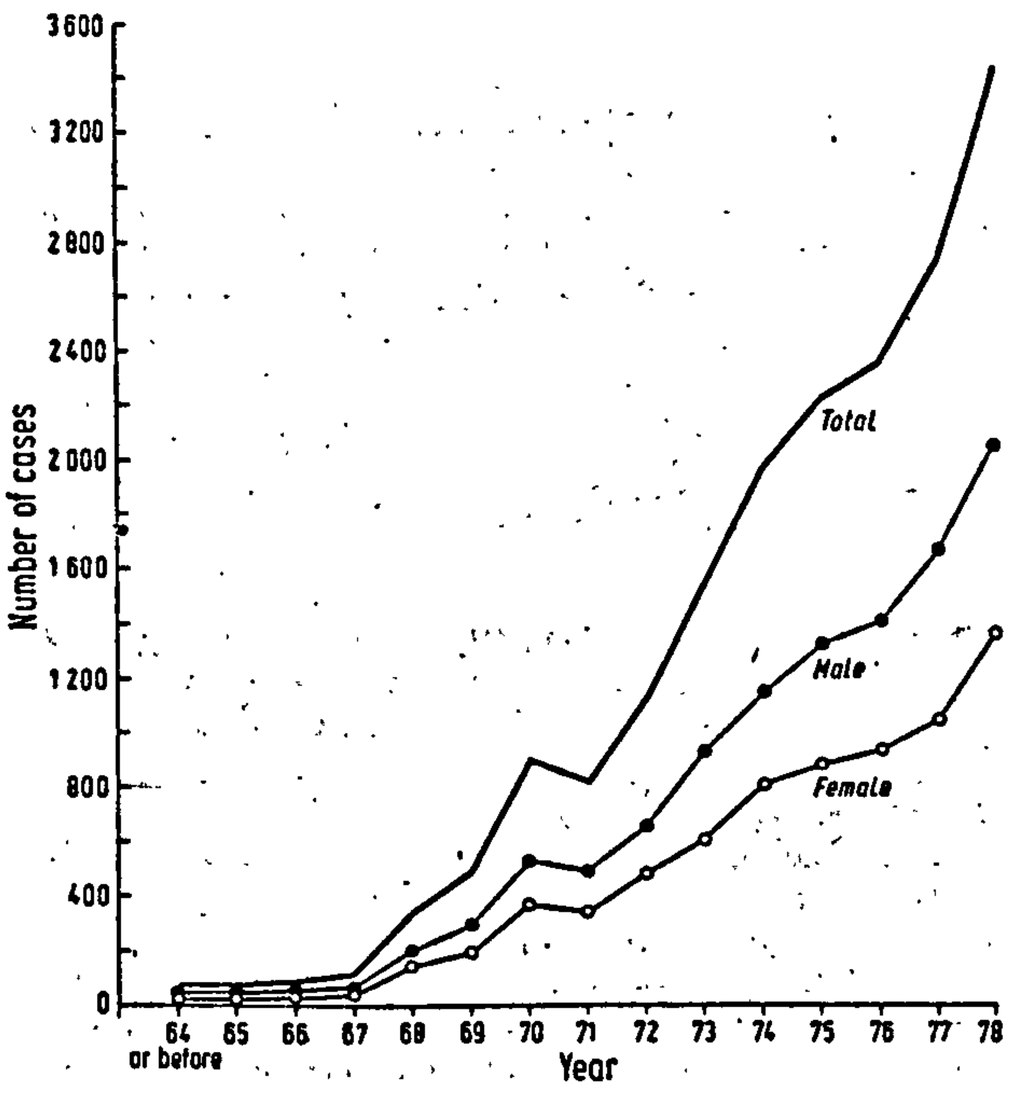
KAWASAKI DISEASE: A WORLDWIDE SURVEY

SIR,—Kawasaki disease, an acute febrile, mucocutaneous condition, has become an important disease of infants and young children. About 12 000 cases had been registered throughout Japan by 1976, and there have lately been reports from other countries. This disease causes great concern because 1–2% of those afflicted die suddenly from coronary thrombosis months or even years after apparently complete recovery. Furthermore, more than 60% of survivors examined by angiocardiography have been found to have some abnormalities, including aneurysm in the coronary arteries. The ninth revision of the W.H.O. International Classification of Diseases, which came into effect on Jan. 1, 1979, adopts Kawasaki disease under the separate rubric (446·1) of "mucocutaneous lymphnode syndrome (MCLS)".

A current nationwide survey being done by the M.C.L.S. Research Committee in Japan indicates that the number of the cases continues to increase in this country. More than 6000



Kawasaki disease registered in Japan.

additional cases were reported throughout Japan during 1977–78, resulting in a total of 18 182 cases (10 884 males and 7298 females). Of these cases, 184 were fatal (1·0%). However, the death-rate has lately declined; it was 1·2–2·6% before 1973, 1·0% in 1974, 0·7% in 1975–76, and 0·5% in 1977–78. This decrease may be partly due to changes in therapy such as the use of aspirin, rather than steroids, and coronary-artery surgery.

The male:female ratio is 1·5:1; the disease peaks around 1 year of age; the incidence is higher in April to September and in December to January; no distinct geographical pattern in incidence has emerged.

The Japan M.C.L.S. Research Committee is undertaking a worldwide postal survey to clarify the ætiological features of the disease would appreciate it if *Lancet* readers would kindly provide us with information on their experience of the disease outside Japan. We can provide diagnostic guidelines with colour illustrations if asked.

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SCREENING FOR CERVICAL CANCER

SIR,—Although the death-rate from cervical cancer of women below the age of 35 is rising in the UK, the total number of deaths is small.[1] The recommendation by the British Society of Cytologists, that screening below the age of 30 years[2] be selective seems to have been determined by the cost and effort of screening millions of women when set against the benefit of saving this small proportion of lives. We feel that age-specific incidence rather than age-specific mortality is the better criterion of assessing the problem of cervical carcinoma in younger women. As part of a survey into the association between cervical carcinoma and the herpes type 2 virus in patients attending St Thomas' Hospital, we found the incidence of premalignant and malignant cervical lesions in this age-group to be high. 39 of 166 (23%) patients with dysplasia, carcinoma-in-situ, and invasive carcinoma were aged 30 years or younger. Of these, 23 had dysplasia, 11 had carcinoma-in-situ, and 5 had invasive carcinoma—i.e., 16 (14%) of a total of 114 patients requiring immediate treatment (patients with carcinoma-in-situ and invasive carcinoma) were 30 years or younger. 8 of 39 (21%) patients below the age of 30 years were West Indian in origin. Approximately 80% of both West Indian and Caucasian patients in this age-group were in social classes I-III.

36 of 70 patients who presented with clinical invasive carcinoma were aged 50–70. Since the time for a tumour to evolve from a premalignant to a malignant stage has not been established, and may well be dependent on many unknown factors, we disagree with Melcher and Linehan[3] that screening is no longer necessary in symptom-free postmenopausal women who have previously had a negative smear.

Although a cervical screening programme has been carried out in the UK for over 10 years, the incidence and mortality from cervical cancer in the UK has not decreased appreciably, and it would be of interest to know what proportion of the population at risk are in fact being screened. In British Columbia[4] where all sexually active women above the age of 20 were screened, a 33% reduction in incidence rate was achieved in 17 years. In Toledo, USA[5] where, over a period of 19 years, the proportion being screened was stepped up gradually from approximately 15% to 90% of those at risk, a 66% reduction in the incidence rate was achieved. Similarly, in Louiseville, Kentucky,[6] where screening began in 1956, 94% of the population at risk were being screened by 1967. A 57% decrease in incidence rate and a 51% decrease in mortality was observed from 1955 to 1973. In contrast, in Auckland, where only some 20% of the women at risk were being screened, the incidence and mortality rates which were already falling before the campaign, did not show an accelerated response.[7]

There are basically two criteria by which the cost benefit of widespread cervical screening and thereby the detection of early cervical lesions may be assessed—the benefit to the patient and the benefit to the health services. The treatment of early invasive carcinoma involves extensive surgery or radiotherapy which is often debilitating and which always castrates. Thus, though the cure-rates in early cervical carcinoma are good, the price is high. Compared with this, the benefit to the patient of a simple curative procedure applied to preinvasive lesions which involves the removal of selected areas of abnor-

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mal cervical epithelium, are only too obvious. In terms of the expenses incurred by the health services in treating the more advanced lesions, it was estimated in the U.S.A. that for every dollar spent, 9 were saved.[8]

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RISK OF MYOCARDIAL INFARCTION IN ORAL-CONTRACEPTIVE USERS

Sir,—Dr Jick (June 2, p. 1187), commenting on our study[1] of oral contraceptives (OCs) and myocardial infarction, argues that our study was seriously biased, but we find his reasons unpersuasive.

Avoidance of OCs by women with predisposing conditions such as angina or diabetes—together with the rarity of these conditions—does mean that our study "cannot provide information on the hazards of OC use in such women." We agree, and so stated. By the same token, Jick's speculation about the possibility that OC use might depend on the severity of a predisposing condition such as hypertension seems moot.

We are not familiar with any study, or theory, connecting the "coronary-prone" personality type to "the decision to continue or discontinue OCs in the face of repeated warnings." For this reason, personality type, as well as many other familiar risk indicators of myocardial infarction, were not discussed as potentially confounding variables.

Jick draws attention to our figure of 539 controls without predisposing conditions. This was a transcribing error which we regret. In fact, there were 539 controls with, and 1203 without, predisposing conditions. Thus the proportion of controls without predisposing conditions was 69%, a figure that accords well with those in other studies.

Jick gives no reason—and we cannot think of any—why, among women not using OC's, those protected from pregnancy by sterilisation should have been excluded (while users of intrauterine devices, diaphragms, and so on, were acceptable). He even suggests that women whose husbands had had vasectomies should have been excluded, as if a husband's contraceptive practice could be a risk factor for myocardial infarction in the wife: we saw no reason to make allowance for vasectomy and hence we did not record data on it. However, we did record data on tubal ligation. Exclusion of women who had tubal ligations did not materially change any of the estimates of OC effect upon myocardial infarction risk. Nor did the further exclusion of predisposed women, together with women over 45 years of age, appreciably diminish the discordance between our results and those of Jick et al.:[2,3] from the data in the accompanying table the rate ratio among women below the age of 45 was 6·4, whereas in Jick's study the corresponding estimate among women below the age of 46 was 15.

In attempting to explain the discordant results between our study and his, Jick concentrates on what he sees as possible biases in our study but does not respond to our reservations about possible selection bias in his own investigation. When we sought permission to interview patients we frequently found the physician and patient eager to collaborate if OCs were in use at the time of the infarction; conversely, we were often told that it was unnecessary to proceed with the interview if OCs were not in use. Such a tendency is conducive to an upward bias in the exposure-rate among the enrolled cases and, thereby, in the magnitude of the observed association. For this reason we aimed at a 100% participation-rate within a pre-defined sampling frame. We achieved a rate of 94%, while Jick was able to interview only 15% of the potential cases considered eligible for study. We continue to be concerned that the participation of such a small subgroup might have been enhanced by knowledge, on the part of the consenting persons, of OC use.

RELATION OF MYOCARDIAL INFARCTION TO RECENT* OC USE, PREDISPOSING CONDITIONS† AND TUBAL LIGATIONS EXCLUDED

Age (yr)	Category	No. of women	No. of users
25–29	MI	4	3 (75%)
	Control	203	54 (27%)
30–34	MI	7	5 (71%)
	Control	257	26 (10%)
35–39	MI	11	3 (27%)
	Control	196	23 (12%)
40–44	MI	23	4 (17%)
	Control	171	6 (4%)
45–49	MI	33	3 (9%)
	Control	172	4 (3%)

*Last use within the month before admission.
†Defined as: ponderal index (weight [lb]/height [in]²) ≥0·04 (1 lb=454 g; 1 in=2·54 cm); treated diabetes; history of lipid abnormality; treated hypertension; treated angina pectoris; history of pre-eclamptic toxæmia.

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HEPATOCELLULAR CARCINOMA IN A PATIENT ON ORAL CONTRACEPTION WHO HAD BEEN EXPOSED TO HEPATITIS B VIRUS

Sir,—Hepatic tumours in patients on oral or systemic contraceptives have been reported.[1] Usually they are benign adenomas,[2,3] and primary hepatocellular carcinoma (PHCC) is rarer. However, in endemic regions (especially in Africa and Asia) where hepatitis B virus (HBV) is prevalent, the incidence of PHCC is also high,[4] and over the past decade oral contraception has been introduced into many developing countries. We report here the earlier than usual development of PHCC in a patient who had evidence of previous asymptomatic HBV exposure and who was on the pill at the same time.

The patient, a female Chinese, age 29, presented in January, 1979, with a 2-month history of pruritus, epigastric discomfort, and weight loss. There was no history of hepatitis or liver disease. In 1972 she started taking 'Anovular' (norethisterone acetate 4 mg, ethinylœstradiol 50 μg) and she had been on 'Ovral' (levonogesterol 0·5 mg and ethinylœstradiol 50 μg) since 1974. She was emaciated. There were no signs of chronic liver disease. The right and left lobes of the liver were enlarged to 16 cm and 15 cm, respectively. Spleen was 3 cm enlarged. A bruit over the right lobe was present. The results of investigations were: bilirubin 1·2 mg/dl, alkaline phosphatase 184 units (normal <100), SGOT 135 IU/l (normal <40), SGPT >200 IU/l; α_1-antitrypsin 538 mg/dl (130–320), α_1-acid-glycoprotein 180 mg/dl (45–110); hepatoma liver antigen positive;[5] α-fetoprotein 1100 ng/ml, HB_sA_g negative counter-immunoelec-

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